REMARKS

Claims 1 and 3-16 are pending. Claims 13-16 have been withdrawn from consideration.

35 U.S.C. 103 obviousness rejection – Secondary considerations

The examiner has rejected the current claims under § 103 as being obvious over a combination of prior art references. While applicant contends that the combination of references cited by the examiner do not in fact support a § 103 rejection for the reasons discussed below, more to the point is the fact that, powerful secondary considerations are extant that strongly militate against a finding of obviousness. These secondary considerations include, without limitation, a long-felt, unfulfilled need for the invention and the express failure of others to fill that need. That is, the examiner is directed to and requested to consider the following:

1. Guttman, D., et al., "Multiple Infections of *Ixodes sapularis* Ticks by *Borrelia burgdorferi* as Revealed by Single-Strand Conformation Polymorphism Analysis," J. Clinical Microbiology, **1996**, 652-656. In this paper, ticks infected with four strains of *B. burgdorferi*, arbitrarily designated mobility classes 1 – 4, we analyzed. On page 654 of the Results, the authors state that "From the DNA sequencing, there is no way to show that MC3 is in the sample when both MC1 and MC4 are present."

Thus we have here an early indication of a need or desire to be able to identify multiple infective strains in an individual by DNA sequencing and an express statement of failure to fulfill that need, the failure further demonstrating the difficulty in doing so, which further suggests that at the time, 1996, those skilled in the art would have been very skeptical that DNA sequencing could achieve the desired goal.

2. Jacobs, M. V., et al., "Reliable high risk HPV DNA testing by polymerase chain reaction: an intermethod and intramethod comparison," J. Clin. Pathology, **1999**, 52:498-503. In this reference, humanpapillomaviruses, HPVs,

is specifically addressed. On page 502, under "Intermethod comparison" it is stated that

> Direct sequencing of GP5+/6+ PCR products apparently failed to identify under-represented HPV types in the multiple HPV infections"

once again at last impliedly indicating that a need or desire to identify distinct strains of HPV existed and that the need was unmet; specifically by use of DNA sequencing.

3. Gharizadeh, B., et al., "Typing of Human Papillomavirus by Pyrosequencing," Laboratory Investigations, **2001**, 81(5) 673-79. In this paper, pyrosequencing, which at the time was a relatively new technique (Ronaghi, et al, 1998), was for the first time compared to conventional DNA sequencing techniques to genotype HPVs. The same GP5+/6+ primer previously used by Jacobs, et al., was employed. On page 677 of the report, the authors stated:

> As with other available methods; multiple infections present in one specimen might be problematic to detect, depending on the proportional dominance and number of genotypes present in the amplicon. At present, pyrosequencing might not be particularly useful for identifying infection with more than one HPV genotype because multiple infections give sequence signals from all of the available types on the specimen. Typing may be possible provided one type is solidly dominant, with low background signal(s) from other existing genotypes. However, this information alone may be insufficient.

Here, then is another implicit indication of the desirability to be able to detect multiple infections in one subject and an express statement that the authors – and therefore those skilled in the art - doubted that such could be accomplished by DNA pyrosequencing.

4. van Doorn, L-J, et al., "Molecular detection and genotyping of human papillomavirus," Expert Rev. Mol. Diag., **2001**, 1(4):394-402. This review, published two years prior to the filing date of the current application corroborates Gharizadeh's position:

> Rapid sequencing methods are now becoming available for high-throughput to permit application in routine analysis of clinical samples. However, it should be noted that sequence analysis is not very sensitive to simultaneously detect different sequences in a mixture. Sequences only representing a minority of the total PCR product may easily remain unnoticed and only the predominant genotype will be detected. This may be insufficient to analyze clinical samples containing a mixture of different HPV genotypes and will underestimate the prevalence of infections with multiple HPV genotypes, which has important consequences... (Page 397.)
>
> The presence of multiple HPV genotypes is a common phenomenon in some patient groups. Up to 35% of HPV-positive samples form patients with advanced cytologic disorders and more than 50% of HIV-infected patients contain multiple HPV genotypes... (Page 397.)

This review unambiguously states the problem, implies that a need exists to solve it given the percentage of patients so infected, and further expressly notes that it could not be solved using DNA sequencing.

5. Wall, S. R., "Cervical human papillomavirus infection and squamous intraepithelial lesions in rural Gambia, West Africa: viral sequence analysis and epidemiology," British, J. Cancer, **2005**, 93:1068-76. In this paper, which, significantly, was published at least two years after the 2003 filing date of the present application, the need to be able to separately determine strains of HPV is once again expressly set forth:

> HPV DNA sequence must therefore be defined to ensure vaccine efficacy and present selective emergence of rare, virulent variants." Page 1068.

On page 1070, however, the authors state:

> These samples were sequenced with the MY 09/11 primers; five had either degenerated in storage or would not amplify, two contained multiple templates and were impossible to sequence [exactly what the current invention is directed to] ... (emphasis added).

Thus, in 2005, the need was still present, it was still unmet and DNA sequencing was becoming less and less appealing as a means of solving the problem to those skilled in the art.

6. Giuliani, L., et al., "Comparison of DNA sequencing and Roche Linear Array® in human papillomavirus (HPV) genotyping," Anticancer Research, **2006**, 26:3939-41. This article once again emphasizes that DNA sequencing was considered at the time, three years after the filing of the current invention, as not being useful for determination of multiple infections:

> Conclusion: The Roche Linear array® [a non-sequencing technique] is a highly accurate assay for HP genotyping. This is particularly true in the presence of multiple infections **which DNA sequencing is unable to resolve**. (Emphasis added.)

7. Lee, S. H., et al., "Routine human papillomavirus genotyping by DNA sequencing in community hospital laboratories," Infectious Agents and Cancer, **2007**, 2:11. Here, 6 years after the filing date of the current application, a DNA sequencing method capable of determining multiple infections in a single reaction still constituted a highly desirable and diligently sought after need that as of this time was considered unmet:

> Among the 107 nested PCR-positive samples, DNA sequencing with the GP6+ consensus general primer yielded multiple overlapping unreadable sequences in 5 cases. Using the individual type-specific primer sequencing for HPV-6 , -11, -16 and -18 proved that one of them contained HPV-16, but not the other three genotypes, and that one contained a mixture of HPV-16 and HPV-18, but not the other two genotypes. For the remaining 3 mixed infection samples, repeated individual DNA sequencing failed to produce a readable primer extension/termination reaction with any of the four type-specific primers. Therefore, these 3 latter cases were considered to be multiple infections caused by HPVs other than the four vaccine-relevant types and grouped under the 'low-risk" category. (Page 6 of 11, emphasis added.)

It is simple beyond question that as recently as one year ago there was no DNA sequencing technique, pyrosequencing or otherwise, that was capable of determining multiple infections. The above citations establish that the need for such a technique was felt for at least 10 years and was clearly unmet. It further is evident that numerous groups of skilled artisans had tried to find such a sequencing technique and had failed. Thus, the secondary considerations alone militate strongly against any assertion that the present technique would be found obvious to those skilled in the art. In fact, it is also fair to say that the abject failure of so many to find a DNA sequencing technique that could simultaneously identify multiple infective agents in a single subject was so pervasive that the art was unquestionable teaching away from the current invention, i.e., it was say in effect, stay away DNA sequencing will not work.

The examiner is requested to reconsider and withdraw the rejections based on the above secondary considerations alone. The following is presented to point out that, secondary considerations aside, the art cited by the examiner does not in any event render the current invention obvious.

35 U.S.C. § 103 Rejection of claims 1, 3-6 and 17

The examiner rejected claims 1, 3-6 and 17 as being unpatentable over Alderborn, et al., Genome Res., **2000**, 10(8):1249-58, in view of Ronaghi, Anal. Biochem., **2000**, 286(2):282-8 and, further, in view of Caskey, et al., U.S. Pat. No. 5,582,989.

In the examiner view, Alderborn teaches determination of single-nucleotide polymorphisms (SNPs) using real-time pyrophosphate DNA sequencing by providing sample containing a target DNA with variable sequence regions, amplifying the DNA, providing sequencing primers, hybridizing the primers to the target DNA and sequencing the variable regions by pyrosequencing.

The examiner admits that Alderborn does not teach using a mixed pool of structurally different primers, each primer being specific for one species, group or target and mixing such mixed pool of primers with a target DNA. The examiner notes, however, that Alderborn teaches identification of multiple SNPs on a

single target DNA using one primer and then opines that this makes it clear in the art that multiple SNPs could be resolved in one reaction.

The examiner next opines that, insofar as using two structurally different primers to resolve multiple SNPs sites is concerned, Ronaghi teaches that even after addition of single-stranded binding protein to a pyrosequencing procedure, the read-length is limited to about 30 nucleotides.

The examiner also states that the extension of multiple primers, each primer drawn to a different target sequence in the same reaction, which the examiner characterizes as multiplex extension, was a well-known concept at the time the current invention was filed and then cites Caskey as highlighting the advantages of multiplexing nucleic acid amplification using structurally different primers, including the ability to detect multiple different separated target sequences as well as the ability to dectect multiple different loci of the same target sequence separated by large sequences in between the loci.

Specifically as to claims 3 and 4, the examiner opines that Caskey teaches multiple different target DNA from virus and bacteria.

Likewise, with regard specifically to claims 5 and 6, the examiner opines that Caskey teaches multiple different disease-linked variants.

Based on the above, the examiner concludes that it was clear at the time of the invention that multiple different target sequences could be extended in a polymerase extension reaction and that it therefore would have been prima facie obvious to a skilled artisan at the time of the invention to use multiple structurally different primers within the same reaction for detection of multiple different SNPs separated by large sequences, i.e., multiplex SNP detection, since the prior art demonstrates that such primers can extend different target loci separated by large sequences and that the skilled artisan would have a reasonable expectation of success since the prior art demonstrates that pyrosequencing can identify multiple different SNP sites within the same reaction.

Applicant traverses.

Applicant's response

First it is noted that Alderborn has nothing whatsoever to do with identifying multiple infections by different species in an individual. All Alderborn did was compare two alleles of the same gene to determine whether one of them contained one or more single nucleotide polymorphisms , which he determined by using a simplistic pattern recognition procedure. That is, the patterns of the two alleles would be more alike than different, differing only if a SNP was present. If the genes had been derived from two different species, it is evident that Alderborn's pattern recognition would be incapable of reveal anything.

Furher, as the examiner freely admits, Alderborn does not teach using a mixed pool of structurally different primers, each primer being specific for one species, group or target and mixing such mixed pool of primers with a target DNA. Neither, however, does Caskey. Caskey does indeed use more than one primer; however, as the examiner notes, Caskey is performing a standard multiplex PCR reaction as described initially by Chambelin, et al. in 1988. That is, multiplex PCR as practiced by Caskey does indeed enable simultaneous amplification of many targets of interest in one reaction using more than one pair of primers. The targets of interest, however, comprise different locales on one and the same strand of DNA to educe multiple deletions, mutations and/or polymorphisms in that particular DNA. Multiplex PCR as initially described and as practiced prior to the present invention simply could not be used to identify multiple different strains in a single sample as the references cited above clearly indicate. That is, amplification and extension is not synonymous identification due to probably biases, which is why multiplex PCR is most often combined with other techniques for accurate identification.

The examiner is requested to reconsider and thereupon withdraw the rejection.

35 U.S.C. § 103 rejection of claims 7-12

The examiner has rejected claims 7-12 under § 103(a) as being unpatentable over Aldeborn, et al., in view of Ronaghi, Caskey, et al. and, further In view of Rader, et al., "Type-specific primer-mediated direct sequencing of

consensus primer-generated PCR amplicons of human papillomavirus: a new approach for the simultaneous detection of multiple viral type infections," J. Virol. Methods, **1995**, 53(2-3):245-54. The examiner refers back to the arguments posed in the above rejection insofar as Alderborn, Ronaghi and Caskey are concerned but then notes that none of those articles specifically teach the sequencing of HPV. The examiner then notes that Rady teaches amplification of a conserved region within multiple different HPV types and subsequent sequencing with sequence-specific primers, materials and methods. That examiner states that, with regard to claim 11, sequencing of low yield amplification of fragments is inherent to the methods of Ye. Then, the examiner argues that would have been *prima facie* obvious to a skilled artisan at the time of the current invention to use the methods suggested by the prior applied references to detect certain HPV types within a sample since the prior art demonstrates such methods are capable of identifying many different nucleic acid templates within the same reaction. Finally, with regard to claim 12, the examiner argues that Caskey teaches the importance of designing primers that do not anneal to unspecified sequences and that it would therefore have been obvious to the skilled artisan at the time of the invention to design multiplex sequencing primers such that they do not anneal to unspecified sequences and thereby provide erroneous sequencing information.

Applicant traverses.

Applicant's response

As discussed above, at the very most, all that Alderborn, Ronaghi and Caskey teach up to the time of the present invention is multiplex PCR to reveal multiple deletions, insertions, mutations and/or polymorphisms in a single strand of nucleic acid taken from a single gene. There is nothing in their disclosures to even remotely render the present invention obvious. Rady does nothing to remedy the situation. All that Rady teaches is HPV amplification with consensus and degenerate GP and MY primers and then sequencing the amplicons with the general primer by Sanger dideoxy sequencing. Rady does not use any multiple specific sequencing primers and does not, and cannot, detect multiple infections.

The PCR cannot differentiate between different HPV types and the described DNA sequencing cannot differentiate between multiple HPV infections. There is nothing in Rady to suggest otherwise.

The examiner is requested to reconsider and thereupon withdraw the rejection.

CONCLUSION

Based on the above remarks, in particular the secondary considerations discussed above and which virtually eliminate any possibility of obviousness, applicant believes that this application is in condition for allowance and respectfully requests that it be passed to issue.

A one month extension of the time for filing this response is requested and the Commissioner is authorized to charge the fee due to Squire, Sanders & Dempsey Deposit Account No. 07-1850.

Date: 25 September, 2008

Respectfully submitted,

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Multiple Infections of *Ixodes scapularis* Ticks by *Borrelia burgdorferi* as Revealed by Single-Strand Conformation Polymorphism Analysis†

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The genetic heterogeneity of the spirochete *Borrelia burgdorferi* within single adult black-legged ticks from Shelter Island, N.Y., was determined by cold, single-strand conformation polymorphism (SSCP) analysis. The central region of the *ospA* gene of *B. burgdorferi* from infected ticks was amplified by nested PCR. Amplified product of the correct size was obtained from 20 of 45 ticks (44%). This is the fraction of ticks that is expected to be infected with *B. burgdorferi*. Four variant classes were determined by SSCP analysis. Eight ticks were infected with a single variant, nine ticks were infected with two variants, two ticks were infected with three variants, and one tick was infected with all four variants. DNA from each variant was sequenced. Five different sequences were found. The sequence of each variant was different from that of another variant by a single base. SSCP analysis could distinguish three of the four single-base changes found in the region.

Lyme disease is a tick-borne illness caused by the spirochete *Borrelia burgdorferi* (4). Studies using various molecular and immunological typing systems have revealed a large amount of genetic variability within the species (3, 16). Consequently, the *B. burgdorferi* sensu lato species complex can be divided into a number of different genospecies, four of which have been formally designated: *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. japonica* (3, 26). Additional strains which cannot be placed in one of these genospecies have been identified (21). The first three genospecies have been isolated from humans and are pathogenic. The others have been isolated from ticks of the genus *Ixodes* or mammals other than humans. Of the pathogenic genospecies, *B. burgdorferi* sensu stricto alone occurs in the United States, while in Europe all three occur.

The immune response to *B. burgdorferi* is represented by an early, prominent humoral response to the endoflagellar protein, p41, and a protein constituent of the protoplasmic cylinder, p93 (6). Both proteins are protected from the immune system by an outer membrane whose major components include OspA and OspB in the tick and OspC and OspD in mammals (22). When mice are immunized with a recombinant OspA, they are protected from challenge by the same strain of *B. burgdorferi* (11, 23) but not necessarily from challenge by heterologous strains of *B. burgdorferi* (12, 13).

A phylogenetic analysis was performed on 15 isolates of *B. burgdorferi* sensu lato by using DNA sequences obtained from the chromosomal genes *p41* and *p93* and the *ospA* gene, located on the 49-kb linear plasmid (9). Comparison of the resulting gene trees revealed that there was very little, if any, genetic exchange between *Borrelia* strains. This result showed that the genus *Borrelia* has a clonal population structure. The analysis also revealed a number of strains which were so highly divergent that vaccines developed against one are unlikely to confer resistance against others. Thus, determination of the genetic as well as the geographic variability of *B. burgdorferi* is vital for the development of efficient vaccines. Successful vaccines will also depend upon understanding how this variability influences cross-reactivity between the different *Borrelia* strains.

Lyme borreliosis presents itself in humans as a multisystemic disorder. Clinical symptoms can vary from an acute skin rash (erythema migrans) to severe dermatologic, arthritic, rheumatologic, cardiac, and neurologic manifestations (24, 25). Genetic variation within *B. burgdorferi* sensu lato has been shown to be responsible for some of the variable symptomatology of Lyme disease (1). Evidence suggests that infections caused by *B. burgdorferi* sensu stricto tend to lead to arthritic symptoms, while those caused by *B. garinii* and *B. afzelii* tend to lead to neurological complications (1, 2). On the basis of the previous findings of genetic and geographic variability in *Borrelia* genospecies and strains (9), it is logical to assume that the variable symptomatology of the disease could be due to variation in *B. burgdorferi* strains. Thus, we report a method that allows rapid screening for genetic variability within a natural population of *B. burgdorferi*.

Natural isolates of *B. burgdorferi* were sampled by collecting the black-legged or deer tick (*Ixodes scapularis*) from Shelter Island, N.Y. This tick is the primary vector for *B. burgdorferi*. A portion of the *ospA* gene of *B. burgdorferi* from infected ticks was amplified by PCR (10). Genetic variation was then surveyed at this locus by single-strand conformation polymorphism (SSCP) analysis, a technique which distinguishes short, fixed-length DNA fragments on the basis of nucleotide substitutions. Minor sequence differences will result in conformational folding differences when the DNA is single stranded. These conformational polymorphisms can then be discriminated by their electrophoretic mobilities on polyacrylamide gels (15). The region flanking the conserved tryptophan at residue 216 or 217 was determined to be hypervariable from a moving window population analysis of OspA from 15 European and North American isolates of *B. burgdorferi* (18). This region of *ospA* was used for determination of variability by SSCP analysis with DNA generated by a novel nested PCR amplification technique from single ticks.

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MATERIALS AND METHODS

Indirect immunofluorescence. Adult ticks were initially surface sterilized in 70% ethanol; this was followed by a 3% hydrogen peroxide wash and three rinses in sterile phosphate-buffered saline (PBS; pH 7.4). Midguts from individual ticks were dissected and triturated in PBS on 1-by-3-in (2.5-by-7.5-cm) microscope slides with Tuberculin syringes, allowed to air dry, and then fixed with acetone for 10 min. These preparations were overlaid with anti-*B. burgdorferi* fluorescein-labeled antibody (catalog no. 02-97-91; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.), and then the mixtures were incubated at 37°C for 45 min. The antibody was diluted within the range of 1:500 to 1:1,000, depending on the optimum resolution on the test slides (slides with diluted, cultured *B. burgdorferi*), prepared fresh each day. The slides were rinsed three times in PBS and were viewed under a fluorescent microscope by using appropriate barrier filters to detect the spirochetes.

Extraction of DNA. Adult black-legged ticks (*I. scapularis*) were collected on Shelter Island, N.Y., and were stored at −20°C. Single ticks were transferred to individual 1.5-ml microcentrifuge tubes and were bisected with a sharpened laboratory spatula. All manipulations were performed with ethanol-flamed sterile instruments. A total of 500 μl of 5% Chelex (Bio-Rad) was added to each tube, and the tubes were rocked at 56°C overnight. After incubation, the tubes were vortexed at high speed for 15 s, heated at 95°C for 15 min, and vortexed for an additional 15 s. Finally, the tubes were centrifuged in a Beckman microcentrifuge for 5 min at maximum speed, and the supernatant (approximately 500 μl) was transferred to a fresh tube.

Nested PCRs. The strategy for the nested PCR amplifications was to use a very low stringency amplification on the first PCR round to generate a heterogeneous population of templates. This heterogeneous collection of templates would then be immediately subjected to a second round of PCR amplification with primers located internal to the first set. The second amplification would be initiated at very high stringency to preferentially amplify the gene of interest and reduce the amplification of spurious templates. A modified "touchdown" PCR (8) amplification strategy was used in later cycles to ensure that product yields were sufficiently high. The two rounds of amplification were performed in the same tube, with no cleaning or preparation of the first-round products before the addition of the second-round reagents.

ospA, encoding an outer surface lipoprotein in *B. burgdorferi*, was studied. The primers were external(+), 5′-AAA AAA TAT TTA TTG GGA ATA GG-3′, beginning at base pair position 4; external(−), 5′-GT TTT TTT GCT GTT TAC ACT AAT TGT TAA-3′, beginning at base position 695; internal(+), 5′-GGA GTA CTT GAA GGC G-3′, beginning at base position 220; and internal(−), 5′-GCT TAA AGT AAC AGT TCC-3′, beginning at base position 564. The *ospA* primer set was designed to amplify a 702-bp fragment in the first round. This fragment would then be used as a template by the two internal primers in the second round. The internal primers amplified a 345-bp fragment.

First-round PCR amplifications used 10 μl of the extracted tick-*Borrelia* DNAs, deoxynucleoside triphosphates at 0.2 mM per nucleotide, Perkin-Elmer Cetus 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.3]), 2.5 mM $MgCl_2$, 2.5 U of native *Taq* polymerase (Perkin-Elmer Cetus), 0.5 μM (each) external primer, and sterile distilled water to a final volume of 50 μl. The reaction mixture was overlaid with approximately 100 μl of mineral oil. The temperature cycling profile was 2 min at 96°C for 1 cycle linked to 30 s at 94°C, 30 s at 37°C, and 2 min at 72°C for 20 cycles. At the end of these 20 cycles the second-round PCR reagents were added to the reaction vessel. The cocktail was composed of deoxynucleoside triphosphates at 0.2 mM per nucleotide, Perkin-Elmer Cetus 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.3]), 2.5 mM $MgCl_2$, 1.25 U of native *Taq* polymerase (Perkin-Elmer Cetus), 1.0 μM (each) internal primer, and sterile distilled water to a final volume of 50 μl. The reaction tube was mixed and briefly centrifuged. The reaction profile for the second round of amplification was 2 min at 96°C for 1 cycle linked to 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C for 10 cycles. The next 10 cycles used an annealing temperature of 55°C; this was followed by annealing at 50°C for 10 cycles and finally 45°C for 5 cycles.

Cold SSCP analysis. *Borrelia ospA* gene fragments, generated by the nested amplification protocol, were surveyed for point mutations by the cold SSCP protocol described by Hongyo et al. (15). A total of 14.6 μl of SSCP loading buffer (0.75% [wt/vol] Ficoll 400, 0.25% bromophenol blue, 0.25% xylene cyanol, 0.68× TBE [Tris-borate-EDTA] buffer) and 0.4 μl of 1 M methylmercury hydroxide (Alfa Aesaer) were added to 5 to 15 μl of the PCR product. The amount of PCR product used depended on the brightness of the band when the PCR product was run on an agarose gel. The mixture was heated at 95°C for 4 min and was then plunged into ice. A total of 15 μl of the reaction mixture was then loaded onto a precast 20% polyacrylamide–TBE gel (Novex), and the gel was electrophoresed in a Novex X-Cell Minicell electrophoresis unit with 1.5× TBE running buffer. The temperature of the lower buffer (which surrounds the gel plates) was maintained at 4 to 8°C by pumping it through a tubing coiled in a refrigerated circulating bath. To obtain the best strand separation, the gel was run for approximately 16 h at a constant 210 V. Shorter running times (4 h at 300 V) were also used, but they were found to give poorer strand separation. Precast 4 to 20% polyacrylamide–TBE gels were also found to give poorer strand separation. The gel was stained with ethidium bromide (5 μg/ml) in TBE for 20 min and was destained in deionized water for 30 min. The bands were visualized under UV light and photographed.

DNA sequencing. DNA sequencing of the PCR product was performed by following the protocol of Khorana et al. (17). The PCR products were electrophoresed in 1% low-melting-point agarose (BRL)–1× TAE (Tris-acetate-EDTA) buffer. The appropriate band was excised from the gel, and double-stranded DNA sequencing was performed directly on the DNA while it was still in the slurry agarose solution by using Sequenase II (U.S. Biochemical). The protocol was modified by the addition of 1 μl of Mn buffer.

Figures. The gels were scanned and analyzed by using Micrographix designer/photomagic 4.0.

RESULTS

The reliability of the nested PCR protocol for amplifying *B. burgdorferi ospA* from ticks was tested by using ticks from the zoonotic mouse colonies at the Centers for Disease Control and Prevention (CDC), Fort Collins, Colo. *B. burgdorferi* B31 is maintained in one colony, with transmission via *I. scapularis* ticks (14, 20). The B31 strain was passaged six times in medium before being inoculated into mice. This infected colony has been maintained for five cycles of infection, in which one cycle consists of passing the infection from mice to ticks and back to mice. Twenty-one ticks which had fed on mice from this colony and 10 ticks which had fed on mice from an uninfected colony were kindly provided by M. Dolan, J. Piesman, and W. Golde (CDC). The ticks from the infected colony are known to have an infection rate of 85 to 95%, while the ticks from the uninfected colony were completely free of *B. burgdorferi* (14). *B. burgdorferi* infection of the ticks was examined by the nested amplification protocol described above. The ticks were processed blind, with respect to their culture of origin, and all PCR reagents were pooled into a single cocktail before dividing them into aliquots and placing them into single reaction mixtures to provide additional controls. The cocktail reagents were added to the PCR tubes prior to the addition of template DNA to further remove any potential of cross-contamination. The results of the nested amplification showed that 0 of 10 ticks from the uninfected colony and 19 of 21 ticks from the infected colony tested positive (data not shown). The observed infection rate of 90.5% is consistent with the expected rate of infection in ticks fed on diseased mice.

Nested PCR amplification of the *ospA* locus was performed on 45 adult black-legged ticks collected from Shelter Island, N.Y. These ticks were collected as questing, unfed adults. The ticks consisted of approximately equal numbers of males and females. Twenty of these ticks gave strong positive results, indicating infection caused by *B. burgdorferi*. Figure 1 shows the results of the nested PCRs from 24 of the 45 Shelter Island ticks. The PCR products were electrophoresed in a 2% agarose gel with a ϕX174 *Hae*III standard. The 345-bp band is clearly visible for 12 of the 24 surveyed ticks. The observed infection rate of 44.4% for Shelter Island ticks is consistent with an infection rate of 40% determined by dissection. Of 76 ticks evaluated by indirect immunofluorescence, 30 were found visually to harbor a spirochete.

Cold SSCP analysis was run with the PCR product from the Shelter Island ticks which were positive for *B. burgdorferi* infection to determine the level of genetic heterogeneity within each tick. The SSCP gel is shown in Fig. 2. At least four strains of *B. burgdorferi* were present in the 20 Shelter Island ticks (Table 1). These strains have been arbitrarily named mobility class 1 (MC1) through MC4. These four mobility classes were found in 65, 40, 40, and 35% of the infected ticks, respectively. A total of 40% of the ticks were infected with a single mobility class of borrelia, while another 45% were infected with two mobility classes. Two ticks (10%) were infected with three mobility classes of borreliae, and one tick (5%) was infected

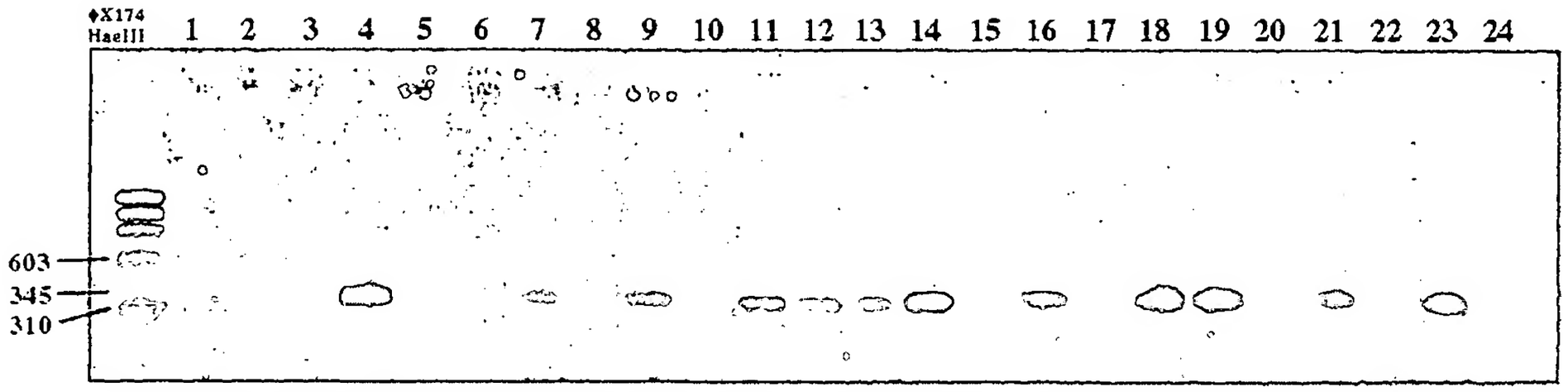

FIG. 1. Nested PCR amplification of a 345-bp fragment from the central region (base pairs 220 to 564) of the *B. burgdorferi ospA* gene from 12 of 24 Shelter Island ticks (lanes 1 to 24, respectively). The ticks in lanes 4, 7, 9, 11 to 14, 16, 18, 19, 21, and 23 are positive, showing the expected 345-bp amplified fragment. The standard (in base pairs) is a ΦX174 *Hae*III digest.

with all four mobility classes. No mobility class was found excessively alone or associated preferentially with other mobility classes. The amplified *ospA* gene segment of *B. burgdorferi* B31, which was originally collected from Shelter Island, was also subjected to SSCP analysis. Its mobility class was MC1.

The amplified segments from ticks which were infected with a single mobility class were chosen for DNA sequencing. DNAs from two isolates of each mobility class were sequenced. The polymorphic sites are listed in Table 2, where the site numbering corresponds to the beginning of the coding sequence. DNA sequencing revealed that MC1 is heterogeneous. The sequences of two selected representatives of MC1 were different from the *B. burgdorferi* B31 sequence, with a T at position 348 instead of the expected C. Other ticks contained an MC1 strain similar to B31. Therefore, MC1 has been broken down into two cryptic subclasses, MC1a and MC1b. Strain B31 belongs to MC1a, while strain CA7-CA7 (5) belongs to MC1b. Strains N40 (9) and 42373-NY3 CA7 (5) were found to belong to MC3 by sequence comparison. No strains were recorded to have sequences that matched the sequences of MC2 and MC4. The sequence of strain ZS7 (9) is similar to that of MC2 except that ZS7 contains an additional change at position 490 and so could have been derived from MC2. The changes at positions 348, 465, and 511 are synonymous changes. Position 511 is a first-position synonymous change of leucine. The change of a G to an A at position 446 changes a glycine to a glutamate.

The amplified segments from ticks infected with multiple mobility classes were also sequenced. Figure 3 shows the double band at position 465 corresponding to the C/T polymorphism. This sequence is from tick 11 (Table 1), which contains mobility classes MC1, MC3, and MC4. There was no polymorphism at position 348, showing that this tick contained MC1a. There was a polymorphism at position 446, a distinguishing characteristic of MC1. From the DNA sequencing, there is no way to show that MC3 is in the sample when both MC1 and MC4 are present, as in this case.

DISCUSSION

The present study shows that SSCP analysis of PCR products encompassing a variable region of the *ospA* gene is a reliable method of rapidly screening for genetic variability within a natural population of *B. burgdorferi* sensu stricto. The basis of the analysis relies on the reliability of the PCR amplification of *B. burgdorferi* DNA from infected ticks. This was tested by the use of ticks from infected and uninfected colonies obtained from CDC. From a blind screening of the ticks, the amplification reactions were negative for all of the ticks from the uninfected colony. An infection rate of 90.5% was observed for ticks from infected colonies. These rates are in agreement with those previously determined by CDC, thus confirming the reliability of the PCR. In other words, the frequency of false-positive and false-negative results by this assay was shown to be insignificant. A further confirmation of the reliability of this assay is that the estimated infection rate of 44% by PCR agrees with the estimated infection rate of 40% by indirect immunofluorescence.

The SSCP analysis of the Shelter Island ticks positive for infection revealed four bands with different mobilities, referred to as MC1 through MC4 in Table 1. In this analysis, each band with a different mobility represents a different strain of *Borrelia* spirochete which has infected the tick under examination. Interestingly, 45% of the infected ticks tested contained two different strains of *Borrelia*, while another 15% contained three or more different strains. The ticks assayed were all questing adult ticks and had two blood meals. Since 15% of the adult ticks were infected with three or more different strains of spirochetes, more than one strain of *B. burgdorferi* must have

TABLE 1. Distribution of the mobility classes in infected ticks

Tick	Presence of the following mobility class:			
	MC1	MC2	MC3	MC4
1	+			+
2	+			
3	+			
4		+		
5			+	+
6	+	+		
7	+	+		
8[a]	+	+		+
9	+			
10[a]				+
11	+		+	+
12	+		+	
13	+			
14	+	+		
15		+	+	
16			+	+
17			+	
18	+	+		
19	+	+	+	+
20			+	
Total	13	8	8	7

[a] The SSCP pattern is not shown in Fig. 2.

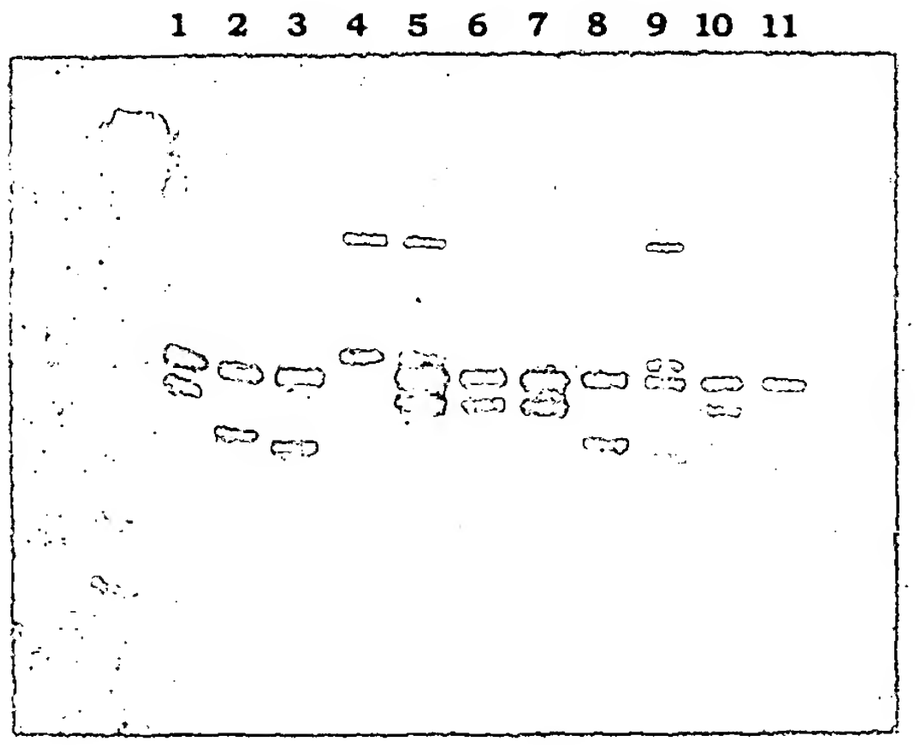

FIG. 2. SSCP gel of the *B. burgdorferi ospA* PCR product from the 20 infected ticks. Lanes 1, 2, 3, and 4, MC1, MC2, MC3, and MC4, respectively; lanes 5 through 11, ticks 1 through 7, respectively (Table 1); lane 12, tick 9; lanes 13 through 22, ticks 11 through 20, respectively. Ticks 8 and 10 (Table 1) do not appear in this figure. The size standards to the right of lanes 1 and 12 are a 123-bp ladder (catalog no. 15613; Gibco BRL). These were not used to measure size, since all of the amplified products are the same size, but were used to check running conditions and relative mobility.

been passed from a mammalian host to a tick during a single blood meal. This shows that the infection in ticks was established by a population of the spirochete rather than a single individual. Preliminary data (unpublished data) from a prospective study following the infection rate in nymphs and adults from the same cohort estimates the infection rate in nymphs to be 25%. If the probability of a tick being infected at each blood meal is the same and independent, then the infection rate in adults would be $1 - (1 - 0.25)^2 = 0.44$, and 6% of the ticks will have been infected twice. This compares with 26% of the ticks being infected with two or more strains.

In order to identify the polymorphisms responsible for the different migration patterns obtained by SSCP analysis, DNAs from representative bands of each mobility class were isolated and sequenced. The sequences from different ticks with the same mobility class were the same (except for the polymorphism found in MC1). This shows that these changes are not mutations arising in the sampled ticks but are polymorphisms in the population. The sequence shows that the amplified segments were from *B. burgdorferi* sensu stricto (9). Nucleotide substitutions (Table 2) were found to be the cause for the differences in mobility. The relationship between these strains is shown in Fig. 4. Each mobility class is different from another by a single mutation. Thus, there are no missing classes. Each mutational change alters a single base, and no class has been lost by genetic drift. This implies either that *B. burgdorferi* maintains a very large effective population size, something very rare in parasites, or that the classes have been maintained by frequency-dependent selection.

TABLE 2. Nucleotide changes defining the different mobility classes

Mobility class	Base at the following nucleotide sites:			
	348	446	465	511
MC1a	C	G	T	C
MC1b	T	G	T	C
MC2	C	A	T	T
MC3	C	A	T	C
MC4	C	A	C	C

The direction of change in Fig. 4 was determined by assuming that the base shared by *B. afzelii* and *B. garinii* is the original or primitive one and that the other one was derived recently by mutation. The T, A, C, and C residues are the primitive bases at positions 348, 446, 465, and 511, respectively. None of the mobility classes described in Table 2 contained only the primitive bases. MC4 contains three primitive bases, and all of the other mobility classes contain fewer. Thus, we have assumed

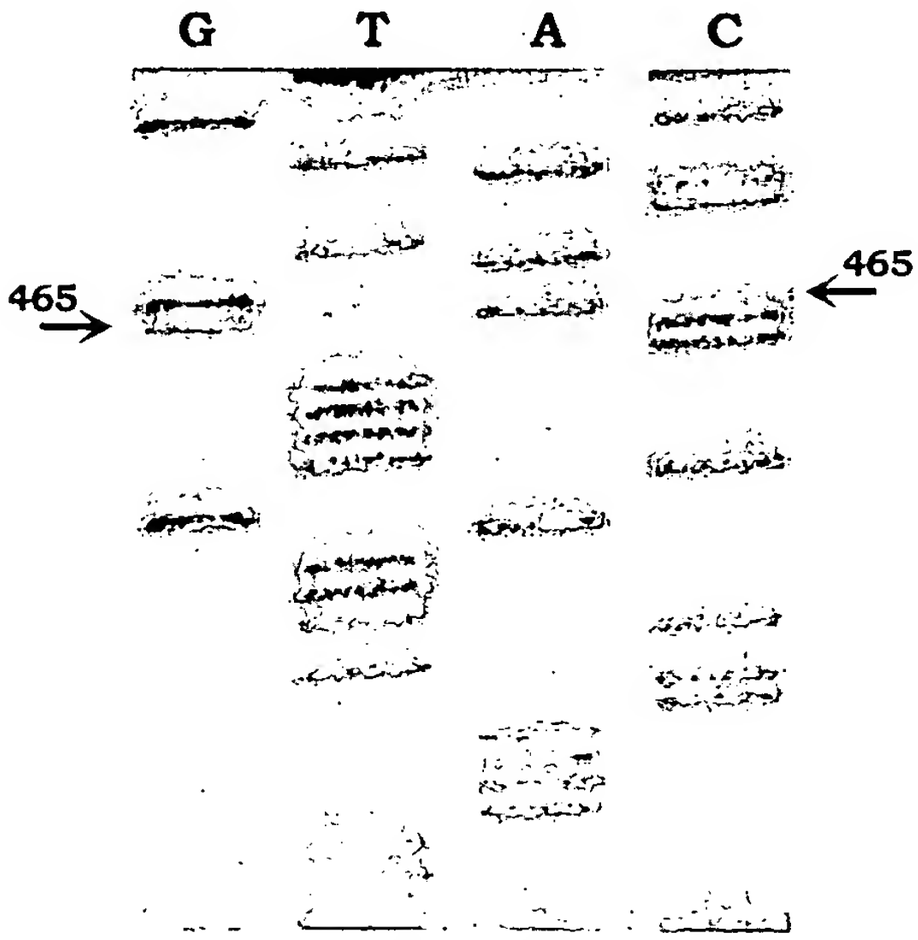

FIG. 3. Sample of the sequencing gel showing infection of a single tick by multiple strains of *B. burgdorferi*. The amplification product from tick 11 was sequenced with the internal(−) primer. A double band can be seen at position 465 corresponding to the T/C polymorphism in Table 2 which is consistent with infection by MC4 and another strain(s) (note that this sequence is off of the minus strand).

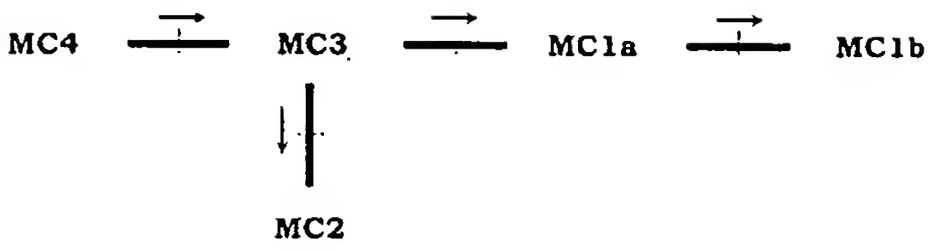

FIG. 4. A dendrogram of the relationships between the *B. burgdorferi* strains. The bars separating the mobility classes (strains) represent single nucleotide substitutions. The arrows point in the direction of the mutational or derived change.

that MC4 is the original type and that the others were derived from it by single mutational events. Thus, we are forced to assume that the T mutated to a C at position 348 in the lineage leading to *B. burgdorferi* sensu stricto and that the T in MC1b is a different mutation or that recombination has given rise to this inconsistency in the pattern of descent. In Europe, the larger amount of genetic variation found in *ospA* across the three species might prevent an accurate assessment of the genetic variation within a single tick, if there are polymorphisms in the regions of primer binding causing differential amplification.

Since the ticks acquire multiple spirochetal strains from a host during a single bite, a high degree of variability in *Borrelia* strains must be maintained in the host population. Mixed infections of different *Borrelia* species have been found in mice in Japan (19) and in humans (7). This aspect of the population biology of *Borrelia* species has not been thoroughly examined to date, but infection by multiple strains could be responsible for the variable symptomatology experienced by patients resulting from a single tick bite. As stated earlier, evidence suggests that the infection caused by different species of *B. burgdorferi* sensu lato leads to different symptomatologies in humans (1). This pattern may be repeated on a more local scale, with variable symptomatology being the result of different strains of *B. burgdorferi* sensu stricto from a single natural population. Therefore, identification of the population of strains of *Borrelia* which exist both in the natural rodent reservoir and in individual ticks would be relevant for the understanding of the variable clinical manifestations of Lyme disease and for the evaluation and development of vaccines against Lyme Disease.

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Reliable high risk HPV DNA testing by polymerase chain reaction: an intermethod and intramethod comparison

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Abstract

***Background*—The development of a reproducible, sensitive, and standardised human papillomavirus (HPV) polymerase chain reaction (PCR) test is required to implement HPV testing in cervical cancer screening programmes and for triaging women with mild to moderate dysplasia.**

***Aims*—To determine the intermethod agreement between different GP5+/6+ and MY09/11 PCR based protocols for the detection and typing of high risk (HR) HPV DNA in cervical smears and to assess the intramethod reproducibility of the GP5+/6+ PCR enzyme immunoassay (EIA) for HR-HPV detection.**

***Methods*—For the intermethod comparison, crude aliquots of 20 well characterised cervical smears comprising five HPV negative samples, and six and nine samples containing single and multiple HPV infections, respectively, were coded and sent from reference laboratory (A) to three other laboratories. One of these (laboratory B) used the GP5+/6+ PCR-EIA and was provided with standard protocols. Another laboratory (C) used GP5+/6+ PCR combined with sequence analysis and type specific PCR, whereas two laboratories (D and E) used MY09/11 PCR followed by restriction fragment length polymorphism (RFLP) analysis for the detection and typing of HR-HPV. The intramethod agreement of GP5+/6+ PCR-EIA was analysed in a subsequent study with four other laboratories (F to I) on crude aliquots of 50 well characterised cervical smears, consisting of 32 HR-HPV positive and 18 HPV negative samples. Standardised protocols, primers, and probes were also provided by the reference laboratory for HR-HPV detection.**

***Results*—In the intermethod comparison, pairwise agreement of the different laboratories with reference laboratory A for the detection of HR-HPV varied between 75% and 100% (κ values: 0.5 to 1). Typing data revealed a broader range in pairwise agreement rates between 32% and 100%. The highest agreement was found between laboratories A and B using standardised protocols and validated reagents. In the intramethod evaluation, pairwise comparison of the laboratories F to I with reference laboratory A revealed excellent agreement rates from 92% to 100% (κ values: 0.88 to 1.0) with an overall sensitivity of 97.5% (195/200) and specificity of 99.5% (199/200).**

***Conclusions*—The detection of HR-HPV as a group is highly reproducible with GP5+/6+ PCR-EIA provided that standardised protocols and validated reagents are used.**

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Keywords: human papillomavirus; polymerase chain reaction; intermethod agreement; intramethod agreement

Worldwide, cervical cancer is one of the most common forms of cancer among women. Although cytomorphological screening of cervical smears (the Papanicolaou test) has reduced the incidence of cervical cancer significantly, the test still has some limitations with respect to sensitivity and specificity. False negative rates for cervical premalignant lesions and cervical cancer between 15% and 50% and false positive rates of about 30% have been reported.[1-3]

To date, it has been shown that high risk human papillomavirus (HR-HPV) genotypes are implicated in the aetiology of cervical cancer.[4] Consequently, the inclusion of HR-HPV testing in cervical cancer screening programmes and the triaging of women with mild to moderate cervical dysplasia has been advocated.[5-8]

As HPV cannot be cultured in vitro and no suitable serological assays are at present available, current methods are based on the detection of HPV DNA in exfoliated cervical cells. These methods include the hybrid capture assay (HCA), a simple direct HPV DNA detection assay using signal amplification,[9] and the polymerase chain reaction (PCR) which is based on the in vitro amplification of target sequences.[10] Both approaches seem robust and potentially suitable for routine. Concerning the PCR based methods, HPV type specific PCR are not suitable for large clinical trials owing to the wide variety of HPV genotypes infecting the genital tract. Broad spectrum detection has therefore been facilitated by consensus PCR assays, with general primers selected from highly conserved sequences of the majority of mucosal HPV genotypes.[11-14] Moreover, subsequent detection steps are continuously amenable to modifica-

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tions to render these general primer PCR assays more feasible for large numbers of samples.

In order to consider HPV testing for cervical cancer screening programmes, issues like the reproducibility between different HPV methods and between different testing centres need further attention. The reproducibility of both HCA and the widely used general primer MY09/11 mediated PCR assay using different read-out protocols has already been evaluated.[15–17] Recently, the read-out system of another widely used general primer mediated PCR system, the GP5+/6+ PCR, has been converted from conventional radioactive Southern blot hybridisation of the PCR products in a colorimetric enzyme immunoassay (EIA) (GP5+/6+ PCR-EIA).[18] Like the latest version of HCA,[19] it is a non-radioactive detection procedure in microtitre plate format which is easy to perform and generates objective numerical data. Although this new GP5+/6+ PCR-EIA system has already been evaluated on clinical specimens,[20] no interlaboratory reproducibility rates are yet available. We therefore assessed the reproducibility of the GP5+/6+ PCR-EIA in a multicentre intermethod and intramethod evaluation. The results of these evaluations are presented in this paper.

Methods

SELECTION OF REFERENCE SAMPLES AND COMPOSITION OF TEST PANELS

Cervical smears were selected from a group of women with abnormal cervical cytology (≥ mild dysplasia) attending the outpatient clinics of the University Hospital Vrije Universiteit in Amsterdam, The Netherlands. The cervical smears were pretreated as described before.[10] Selection of study samples was based on the following criteria:

(1) an adequate quality of the DNA for PCR amplification as determined by a PCR assay with primers spanning 509 base pairs of the β globin gene[21];
(2) the presence or absence of HPV DNA after GP5+/6+ PCR;
(3) confirmation of HPV types by both GP5+/6+ PCR-EIA using HR-HPV oligo (cocktail) probes[19] and HPV E7 type specific PCR assays[22];
(4) samples comprised one or more of the following HPV types considered as high risk: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68.

Fifty cervical smears were selected consisting of 18 HPV negative and 32 HPV positive samples. The 32 HPV positive smears comprised 23 single and nine multiple HPV infections.

For the intermethod comparison study, a subset of the 50 selected cervical smears was used to prepare a test panel of 20 specimens consisting of five HPV negative samples and six and nine samples containing a single and multiple HPV infections, respectively. The HPV positive samples comprised together a diversity of 13 HR-HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 58, 59, 66, and 68).

The test panel for the intramethod comparison study consisted of all 50 selected cervical smears including the 13 different HR-HPV genotypes and in addition HR-HPV 56.

STUDY DESIGN

One laboratory served as the reference laboratory (A) and established the test panel of cervical smears. Aliquots of these specimens (50 µl) were coded and distributed by laboratory A to different laboratories. In the test panels, the HPV negative samples were randomly divided between the HPV positive samples; 10 µl of the study samples had to be used for HPV testing. All participating laboratories had experience with HPV PCR technology. The reference laboratory did not participate in the HPV testing but collected and compared the HPV PCR test results from the different laboratories with its own reference data. The GP5+/6+ PCR-EIA results from the reference laboratory (A) were used as the gold standard as these results were confirmed by an alternative HPV E7 TS-PCR system and were therefore considered conclusive.

INTERMETHOD COMPARISON

Four laboratories (B to E) participated in the intermethod evaluation for the detection and typing of HR-HPV in cervical smears. These four laboratories applied their own in-house HPV PCR assays.

One of these laboratories (B) used the same method as the reference laboratory (A)—that is, GP5+/6+ PCR-EIA with a high risk oligo cocktail probe for the detection of HR-HPV and individual oligo probes for HPV typing and was provided with standardised protocols after an extensive training period.

Another laboratory (C) applied the GP5+/6+ PCR followed by agarose gel electrophoresis to detect the presence of HPV DNA and used type specific (TS) PCR for HPV 6, 11, 16, and 18 combined with direct sequence analysis of GP5+/6+ PCR products in cases of GP5+/6+ PCR positive and TS-PCR negative samples for HPV typing.

Two other laboratories (D and E) used MY09/11 primer mediated PCR. The presence of HPV DNA was analysed by agarose gel electrophoresis of the MY09/11 generated PCR products while typing was performed by restriction fragment length polymorphism (RFLP) analysis and hybridisation of the RFLP products with a generic oligonucleotide probe mixture.

INTRAMETHOD COMPARISON

For the intramethod comparison, four other laboratories (F to I) without previous experience with GP5+/6+ PCR-EIA participated in the studies. The reference laboratory (A) provided a standard operating procedure, digoxigenin labelled high risk oligo cocktail probes, and the GP5+/bioGP6+ primers. The quality of this material was first validated and a sensitivity of between 10 and 200 copies of the HPV genome, depending on the HPV type, could be attained.[17] Other reagents and equip-

Table 1 Intermethod agreement between different polymerase chain reaction (PCR) protocols (B–D) performed in different laboratories and the reference data (A) for the detection of high risk human papillomaviruses (HR-HPV) in 20 cervical smears

Method pair	Agreement obtained for: Positives (n=15)	Negatives (n=5)	% Overall agreement (No of identical / No tested)	κ Statistic
A and B	15	5	100% (20/20)	1
A and C	13	5	90% (18/20)	0.76
A and D	14	5	95% (19/20)	0.88
A and E	10	5	75% (15/20)	0.50

B:GP5+/6+ PCR-EIA using HR (high risk) oligococktail probe for HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. C: GP5+/6+ PCR and agarose gel electrophoresis. D,E: MY09/11 PCR and agarose gel electrophoresis.

Table 2 Comparison between different methods and the reference data for typing of high risk human papillomaviruses (HR-HPV)

Sample	Reference data	Laboratory* B	C	D	E
1	Neg	Neg	Neg	Neg	Neg
2	Neg	Neg	Neg	Neg	Neg
3	Neg	Neg	Neg	Neg	Neg
4	Neg	Neg	Neg	Neg	Neg
5	Neg	Neg	Neg	Neg	Neg
6	18	16, 18	18	18	18
7	39	39	Neg	39	Neg
8	51	51	HPV pos†	51	Neg
9	52	52	52	52	52
10	56	56	56	56	56
11	58	33, 58	58	58	58
Typing agreement compared with reference data for single HPV infections:		6/6 (100%)	4/6 (67%)	6/6 (100%)	4/6 (67%)
12	16, 35	16, 35	Neg	Neg	16, 61
13	16, 68	16, 68	16	16, 68	16
14	31, 35	31, 35	31	35	31
15	33, 35	33, 35	35	35	Neg
16	33, 45	18, 33, 45	45	33, 52	Neg
17	33, 58	33, 58	33	33	33
18	35, 59	35, 59, 66	59	31	31
19	52, 68	52, 68	68	HPV pos†	Neg
20	31, 59, 66	31, 59, 66	66	18	18
Typing agreement per HPV type compared with reference data for multiple HPV infections:		19/19 (100%)	8/19 (42%)	6/19 (32%)	4/19 (21%)
Overall typing agreement compared with reference data:		25/25 (100%)	12/25 (42%)	12/25 (48%)	8/25 (32%)

*Lab B: GP5+/6+ PCR-EIA using individual internal oligoprobes for HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68; Lab C: TS-PCR for HPV 6/11, 16, and 18 and direct sequence analysis of GP5+/6+ PCR products; Lab D, E: restriction fragment length polymorphism analysis of MY09/11 PCR products followed by hybridisation with an oligonucleotide probe mixture.
†The HPV type could not be identified by the typing procedure used, but the sample contained HPV DNA after hybridisation of the PCR products with a general HPV probe (method C) or after agarose gel analysis (method E).
For simplicity of the table, the samples have been sorted according to HPV type.
p Value for differences in typing between single and multiple HPV infections < 0.001 ($\chi^2_{,\ p<0.05}$).

ment had to be purchased from their local distributors.

STATISTICAL ANALYSIS

The intermethod and intramethod agreement for HR-HPV detection was assessed by pairwise comparison of the test results with the reference data using percentage of agreement and the kappa (κ) statistic. κ Values express the proportion of possible agreement beyond chance. A κ estimate of less than 0.4 represents poor agreement, a κ estimate between 0.4 and 0.75 is fair to good agreement, and a κ estimate of more than 0.75 is excellent agreement.[23] For HPV typing, the intermethod agreement was assessed by pairwise comparison of the typing results with the reference data by the percentages of agreement. The χ^2 test was used to indicate significant differences between typing of single and multiple HPV infections.

Results

INTERMETHOD COMPARISON BETWEEN GP5+/6+ AND MY09/11 PCR BASED PROTOCOLS

HPV detection analysis on cervical smears

Aliquots of 20 well characterised cervical smears were subjected to different PCR protocols employed in the different laboratories and compared with the reference data (table 1). Laboratory B correctly identified all 15 HPV positive smears and all five HPV negative samples, resulting in an overall agreement of 100% (20 of 20). Laboratory C failed to detect two specimens of the 15 HPV positive samples, but identified all five HPV negative smears. This resulted in an overall agreement of 90% (18 of 20). Laboratories D and E correctly identified all HPV negative samples, but both laboratories failed to identify one and five HPV positive smears, respectively. This revealed an overall agreement of 95% (19 of 20) for laboratory D and 75% (15 of 20) for laboratory E. The κ values ranged from 0.50 for agreement between laboratories A and E, to 0.76 for laboratories A and C, to 0.88 for laboratories A and D, to 1 for laboratories A and B.

HPV typing analysis on HPV positive cervical smears

Subsequently, to determine differences in HPV typing of six single and nine multiple HPV infections by the different procedures, HPV positive samples were subjected to typing analysis and results were compared with the reference data. The results are shown in table 2.

Evaluation of the single HPV infections showed that laboratory B identified the correct HPV type in all the six samples (samples 6 to 11). However, in two samples (sample 6 and 11) an additional HR-HPV type was detected compared with the reference data. Laboratory C detected the correct HR-HPV type in four of the six single HPV infections (samples 6, 9, 10, and 11). In one single HPV infection (sample 8) the HR-HPV type (HPV 51) could not be identified, while the remaining sample (sample 7) was tested HPV DNA negative. Only laboratory D identified the correct HPV type in all the six single HPV infections (samples 6 to 11) whereas laboratory E correctly identified the HR-HPV type in four of the six single HPV infections (samples 6, 9, 10, and 11). The two remaining samples (samples 7 and 8) were tested HPV DNA negative.

Evaluation of the multiple HPV infections showed that laboratory B correctly typed the HR-HPVs present in all the nine samples (samples 12 to 20). However, in two samples (samples 16 and 18) an additional HR-HPV type was detected compared with the reference data. Only a single HPV type was detected in all nine multiple HPV infections by laboratory C. In all these nine cases the HR-HPV type detected corresponded with one of the multiple HPV types present in the sample according to the reference data. Laboratory D detected single HPV types in five of the nine multiple HPV infections. In three of these (samples 14, 15,

Table 3 GP5+/6+ PCR-EIA results obtained by the different laboratories for the detection of high risk (HR) human papillomaviruses (HPV) in 50 cervical smears compared with the reference data

Sample	Reference data	HR-HPV Lab F	Lab G	Lab H	Lab I
1	–	–	–	–	–
2	–	–	–	–	–
3	–	–	–	–	+
4	–	–	–	–	–
5	–	–	–	–	–
6	–	–	–	–	–
7	–	–	–	–	–
8	–	–	–	–	–
9	–	–	–	–	–
10	–	–	–	–	–
11	–	–	–	–	–
12	–	–	–	–	–
13	–	–	–	–	–
14	–	–	–	–	–
15	–	–	–	–	–
16	–	–	–	–	–
17	–	–	–	–	–
18	–	–	–	–	–
19	16	+	+	+	+
20	16	+	+	+	+
21	16	+	+	+	+
22	16	+	+	+	+
23	16	+	+	+	+
24	16	+	+	+	–
25	18	+	+	+	+
26	18	+	+	+	+
27	18	+	+	+	–
28	31	+	+	+	+
29	33	+	+	+	+
30	39	+	+	+	+
31	45	+	+	+	+
32	51	+	+	+	+
33	51	+	+	+	+
34	52	+	+	+	+
35	52	+	+	+	+
36	56	+	+	+	+
37	56	+	+	+	+
38	58	+	+	+	+
39	58	+	+	+	+
40	59	+	+	+	+
41	59	+	–	+	+
42	33, 35	+	+	+	+
43	16, 68	+	+	+	+
44	33, 45	+	+	+	+
45	35, 59	+	+	+	+
46	52, 68	+	+	+	+
47	16, 35	+	+	–	–
48	31, 35	+	+	+	+
49	33, 58	+	+	+	+
50	31, 66, 59	+	+	+	+

PCR-EIA, polymerase chain reaction–enzyme immunoassay.

and 17), the type detected corresponded with one of the HR-HPV in the sample. In the two other cases (samples 18 and 20), an HR-HPV type was found which did not represent one of the types identified by the reference laboratory. Two multiple HPV infections were detected where in one sample (sample 13) the HPV types were correctly identified and in the other sample (sample 16) the HPV types were partly identified. Of the two remaining samples, one (sample 19) was found HPV positive but could not be identified, while the other sample was scored HPV negative (sample 12). Laboratory E also identified single HPV types in five of the nine multiple HPV infections (samples 13, 14, 17, 18, and 20). In two of these five cases (samples 18 and 20), the HPV type did not correspond with either of the HPV types present in the sample. One double HPV infection was found (sample 12) including HPV 61, which the reference laboratory had not tested for. The remaining three samples (samples 15, 16, and 19) were tested HPV DNA negative.

Taking the typing data together, the percentages of overall agreement with the reference laboratory were 100%, 48%, 48%, and 32% for the methods employed in laboratories B, C, D, and E, respectively. Moreover, the typing agreement of all laboratories together was significantly higher ($p < 0.001$) for single HPV infections (83%; 20/24) than for multiple HPV infections (49%; 37/76).

INTRAMETHOD COMPARISON OF HR-HPV GP5+/6+ PCR-EIA

Crude cell suspensions of 50 cervical smears were analysed in different laboratories by GP5+/6+ PCR-EIA and compared to the reference data.

As shown in table 3, among the HPV negative samples, one sample (3) was tested HR-HPV positive by laboratory I only. Among the HR-HPV positive samples, four samples (24, 27, 41, and 47) were tested false negative. One of these samples (47) was tested HR-HPV negative by two independent laboratories (H and I), while the remaining three samples were scored HR-HPV negative by only one of the laboratories.

Laboratory F correctly identified all 32 HPV positive and all 18 HPV negative specimens of the test panel, while both the laboratories G and H identified all the HPV negative samples but failed to identify one HR-HPV positive sample. Laboratory I correctly identified 29 of the 32 HPV positive samples and additionally tested one sample HR-HPV positive among the 18 HPV negative specimens of the test panel. The percentages of agreement between the different laboratories and the reference laboratory varied from 92% (46 of 50; laboratory I) to 98% (49 of 50; laboratories G and H) to 100% (50 of 50; laboratory F). Likewise, κ values ranged from 0.83 to 0.96 to 1 for laboratories I, G and H, and F, respectively. The results are summarised in table 4.

Table 4 Intramethod agreement between laboratories (E–H) and reference laboratory (A) for the detection of high risk human papillomaviruses (HR-HPV) as a group in 50 cervical smears by GP5+/6+ PCR-EIA

Laboratory pair	Agreement obtained for: Positives (n=32)	Negatives (n=18)	Percent overall agreement (n identical/n tested)	κ Statistic
A and F	32	18	100% (50/50)	1
A and G	31	18	98% (49/50)	0.96
A and H	31	18	98% (49/50)	0.96
A and I	29	17	92% (46/50)	0.83

PCR-EIA, polymerase chain reaction–enzyme immunoassay.

Discussion

In view of potential applications of HR-HPV PCR assays in cervical cancer screening programmes,[24] the intermethod agreement of GP5+/6+ and MY09/11 consensus PCR based protocols was investigated by multiple test centres. A higher reproducibility for HR-HPV detection than for HPV typing was found. Among the different protocols, GP5+/6+ PCR amplification followed by hybridisation of the PCR products with a cocktail probe for HR-HPV types in an EIA format revealed the highest agreement with the reference data. Moreover, excellent intramethod agreement between other test centres was obtained with

this method in a subsequent study. These data indicate that HR-HPV GP5+/6+ PCR-EIA has outstanding reproducibility.

INTERMETHOD COMPARISON

Comparison of different in-house HPV PCR methods with the reference data showed that the agreement was fair to excellent (κ values: 0.5 to 1) for the detection of HR-HPV DNA in cervical smears (table 1). A markedly lower agreement rate (75%) for HPV detection was observed for laboratory E using MY09/11 PCR and agarose gel analysis compared with other laboratories (90% to 100%). It has to be noted that the quality of the DNA in all samples was appropriate for efficient PCR amplification of at least 500 base pair fragments. As the MY09/11 PCR amplifies a shorter fragment of 450 base pairs in the HPV L1 open reading frame, the disagreement cannot simply be explained by inadequate DNA quality of the specimens. This is further supported by the observation that laboratory D, using the same MY9/11 PCR assay including the read-out protocol, obtained an excellent agreement (95%) with the reference data for the detection of HPV DNA. In addition, the agreement between both laboratories (D and E) using MY09/11 PCR was only fair (κ value: 0.40). The discrepancies were restricted to false negative test results. Moreover, since all HPV positive samples included in the test panel contained high amounts of HPV DNA according to the optical density values of the reference data, the false negative test results are also unlikely to be a result of sampling errors.

Furthermore, a broader range in agreement was found for HPV typing compared with the detection of HPV DNA. Moreover, the HPV typing results varied more strongly for the multiple infections compared with the single infections ($p < 0.001$; table 2). In our study it was shown that differences in read-out systems make a large contribution to variations in HPV typing. Direct sequencing of GP5+/6+ PCR products apparently failed to identify underrepresented HPV types in the multiple HPV infections, in contrast to hybridisation of GP5+/6+PCR products with digoxigenin labelled oligo probes in EIA. In addition, this latter method detected some additional HPV types compared with the reference laboratory owing to differences in the interpretation of weak signals. That variations in HPV typing may occur using different protocols is further substantiated by the observation that both laboratories using the same MY09/11 PCR-RFLP procedures had a lower detection rate for multiple HPV infections than the laboratory using GP5+/6+ PCR-EIA. However, in another study,[25] the reverse was found when a similar dot blot procedure was used for HPV typing of both GP5+/6+ and MY09/11 PCR products derived from the same series of samples. These data suggest that the efficiency of HPV testing by consensus PCR is not only dependent on the specificity of the primers but also on the read-out system applied. Moreover, these results strongly reinforce the need for standardisation of read-out systems employed in different laboratories.[26]

INTRAMETHOD COMPARISON

In the intramethod comparison, the reproducibility of the HR-HPV GP5+/6+ PCR-EIA—tested on 50 well characterised specimens—was fairly uniform among different laboratories, as shown by the small differences in the agreement rates (92% to 100%; table 4). Except for one case, the few observed discrepancies comprised false negative test results of HPV positive samples (table 3). Nevertheless, the five false negative test results among a total of 200 tests in the four laboratories show a high overall sensitivity of 97.5% (195/200). Likewise, the specificity of GP5+/6+ PCR for HR-HPV detection was excellent. There was only one single false positive test result obtained among the 200 tests conducted in the four laboratories, resulting in an overall specificity of 99.5% (199/200).

Finally, the main goal of this study was the recognition that HPV testing can be performed reliably by consensus HPV PCR based protocols and between different testing centres. Smits *et al* already showed that agreement between CPI/II PCR and MY09/11 PCR for the detection of HPV DNA in cervical smears was excellent (κ values between 0.82 and 0.84).[27] Moreover, high interlaboratory reproducibilities for the detection of HPV DNA with MY09/11 PCR in clinical specimens of about 88% to 97% have been found previously.[16 17] The results of our study are in line with these reports and suggest that the variation in HR-HPV detection by different consensus HPV PCR based protocols can be quite small. Most importantly, however, testing for HR-HPV as a group appears to be more reproducible than testing for individual HPV types. Since results from recent case–control studies show that the risk for women of getting cervical cancer does not differ significantly for the different HPV genotypes,[28 29] individual HPV typing is unlikely to be more relevant clinically than HR-HPV group specific testing. With the data obtained in this study, this argues that HR-HPV group specific detection should be the strategy of choice in cervical cancer screening programmes. It has additionally been shown that the HR-HPV GP5+/6+ PCR-EIA has high reproducibility for the detection of HR-HPVs and can easily be transferred to other laboratories provided that standardised protocols and validated reagents are used. Therefore, this test could be used in large clinical trials. Recently, a trial of 44 000 women was started to evaluate HR-HPV testing with the GP5+/6+ PCR-EIA in population based cervical cancer screening.

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ARTICLES

Typing of Human Papillomavirus by Pyrosequencing

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SUMMARY: The possibility of using a new bioluminometric DNA sequencing technique, called pyrosequencing, for typing of human papillomaviruses (HPV) was investigated. A blinded pyrosequencing test was performed on an HPV test panel of 67 GP5+/GP6+ PCR-derived amplification products. The 67 clinical DNA samples were sequenced up to 25 bases and sequences were searched using BLAST. All of the samples were correctly genotyped by pyrosequencing and the results were unequivocally in accordance with the results obtained from conventional DNA sequencing. Pyrosequencing was found to be a fast and efficient tool for identifying individual HPV types. Furthermore, pyrosequencing has the capability of determining novel HPV types as well as HPV sequence variants harboring mutation(s). The method is robust and well suited for large-scale programs. (*Lab Invest 2001, 81:673–679*).

Human papillomaviruses (HPV) belong to the *Papovaviridae* family. HPV carry circular double-stranded DNA, approximately 8 kb in length (Godfroid et al, 1998), which encodes for several regulatory and structural proteins known as early (E), E1, E2, and E4 to E7, and late (L), L1 and L2, proteins. These proteins are involved in viral replication and have transforming (oncogenic) properties (Poljak et al, 1998; Schneider, 1993).

More than 100 different HPV types, more than 30 of which infect the cervical mucosa, have been identified on the basis of DNA sequence homology (Chan et al, 1995; Vernon et al, 2000). Because all HPV types are closely related, assays can be designed to target conserved regions of the genome or to target regions whose sequences can best be used to discriminate between different HPV types. Consensus assays use primers directed at relatively conserved regions of the HPV genome. The consensus amplicon is then typed by methods such as dot blot hybridization with type-specific probes, restriction fragment length polymorphism analysis, or gel-electrophoretic DNA sequencing (Vernon et al, 2000).

An alternative approach to specifically typing HPV is sequencing by a new bioluminometric, nonelectrophoretic DNA-sequencing method, called pyrosequencing (Ronaghi et al, 1998). This technique employs a cascade of coupled enzymatic reactions, using DNA polymerase, ATP sulfurylase, and luciferase to monitor DNA synthesis, along with a nucleotide-degrading enzyme in the system enabling iterative nucleotide dispensation to the reaction mixture (Fig. 1). The technique has the advantages of accuracy, flexibility, parallel processing, and simple automation. Additionally, it avoids the use of labeled primers (except in template preparation), labeled nucleotides, and gel electrophoresis.

Because of the high stability and conservation of HPV genomes over evolutionary times (Chan et al, 1995), even short segments of the genome can be used for reliable typing. The nucleotide sequences of the first 50 nucleotide bases of the L1 "consensus region" immediately downstream of the GP5+ primer site are type-specific enough for genotyping of most common genital HPV types. In this report, we discuss the application of pyrosequencing for sequencing 20 to 40 bases of amplicons for HPV genotyping. In this blinded clinical test, we used pyrosequencing to genotype 67 clinical specimens from different individuals. The results were compared with conventional DNA-sequencing data for confirmation. The samples were previously genotyped by type-specific PCR.

Results

An HPV test panel, consisting of 67 samples from different individuals, was amplified in 150 bp fragments from the L1-conserved region in a blinded test. The general primers, GP5+ and biotinylated GP6+,

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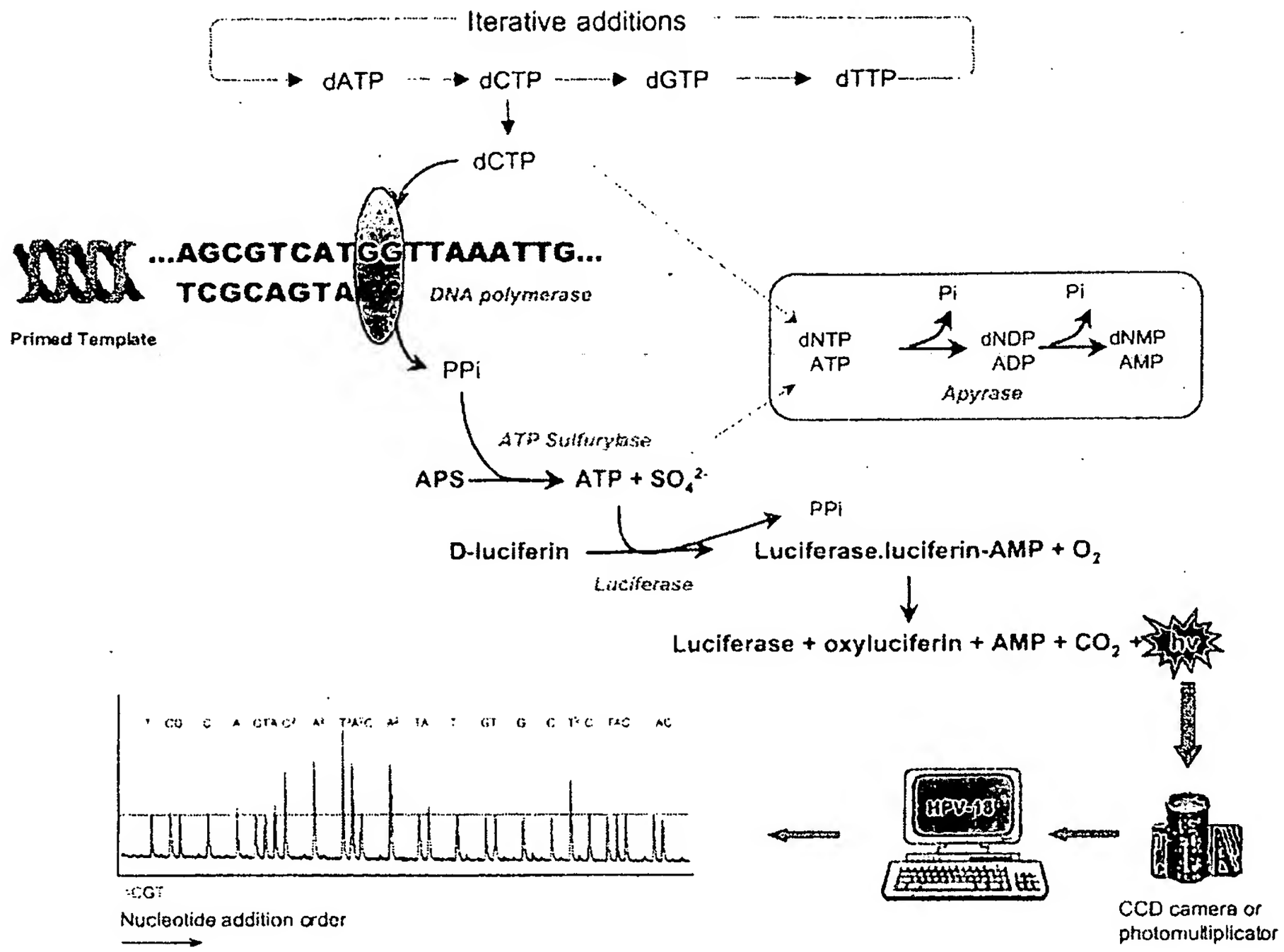

Figure 1.
Schematic representation of the automated pyrosequencing system.

were used. Twelve of the 67 samples were directly amplified from cell lysates, dealt with separately in this report. The other 55 samples were amplified from extracted DNA. The efficiency of the PCR amplification was evaluated by gel electrophoresis and ethidium bromide staining.

The immobilized single-stranded templates hybridized to the GP5+ sequencing primer were sequenced by pyrosequencing to determine the HPV type of each sample. The objective was to obtain sequence data from 20 to 25 bases. As shown in Figure 2, the nucleotide sequences of the first 14 to 40 nucleotides

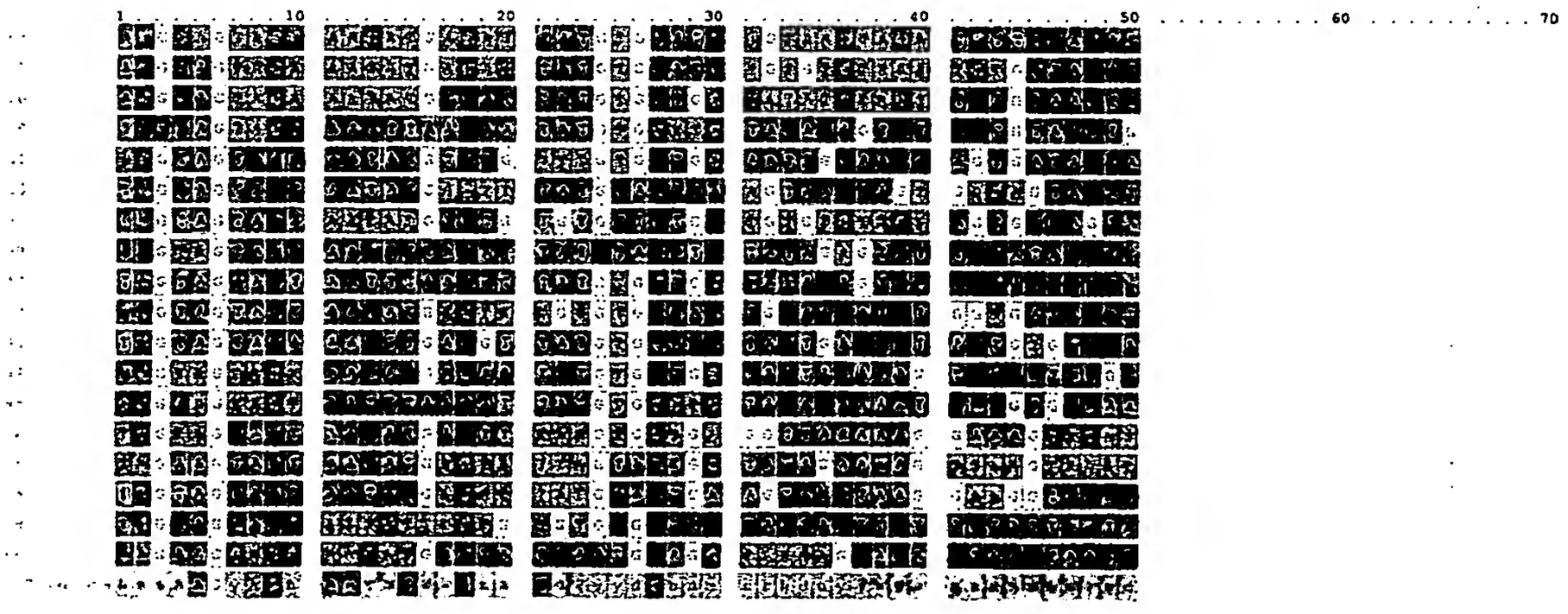

Figure 2.
Color-coded sequence alignment of the first fifty nucleotides downstream of the human papillomavirus (HPV) L1 GP5+ consensus primer site. The alignment was created using the Omiga program, version 1.1.3 (Oxford Molecular Group, The Medawar Centre, Oxford, United Kingdom).

of the L1 consensus region immediately downstream of the GP5+ primer site are sufficiently type-specific among the most common HPV types to allow for typing.

The sequence data obtained by pyrosequencing were analyzed by BLAST search (*http://www.ncbi.nlm.nih.gov/BLAST/*) for HPV genotyping. To confirm the accuracy of the results, the PCR products were also sequenced by conventional DNA sequencing. The samples had also been previously typed by type-specific PCR.

As shown in Table 1, there was 100% concordance between pyrosequencing and conventional DNA sequencing. Figure 3 shows sequence results from seven different HPV types; the height of each peak is proportional to the number of nucleotides incorporated.

All of the 55 DNA extracts amplified with GP5+/6+ were sequenced in 20 to 25 bases by pyrosequencing and typed correctly. Only one sample (IS324, a cellular sample from a healthy individual) required further sequencing. This sample had six HPV type/isolate hits with 18 bases in the BLAST search. The differentiation for IS324 was made with data from 38 sequence bases.

In one case, an amplicon that had been previously (mis)typed as HPV-31 by type-specific PCR, was correctly genotyped as HPV-52 by both pyrosequencing and conventional DNA sequencing Table 1. We also detected a single T to C substitution in HPV-type 31 (Fig. 4).

In the cell lysate samples, only 4 of the 12 amplicons had a single band after gel staining and could be sequenced by pyrosequencing. Mixed pyrosequence signals were observed in the other eight samples. This could be due to unspecific amplifications because multiple bands were observed in the gel staining from those amplicons. Nested PCR was performed on the cell lysate samples using MY09/11 and GP5+/6+ primer sets. All of the nested amplicons were sequenced by pyrosequencing, demonstrating high signal intensity, which indicates large quantities and specific amplification products. The acquired sequences from the nested PCR agreed with conventional DNA sequencing.

In the samples from cervical cancer patients, HPV-16 was found in 20 of 35 cases (57%), HPV-18 in 6 of 35 (17%), and HPV-31 in 3 of 35 (9%). The remaining six samples contained the following HPV types: 6, 33, 35, 45, 52, and 59. Of the samples from dysplastic individuals, HPV-16 was found in 3 of 11 cases (27%), HPV-31 in 3 of 11 (27%), HPV-18 in 2 of 11 (18%), and HPV-66 in 1 of 11 (9%). The following HPV types were found in the samples from screened healthy individuals: HPV-31 in 6 of 21 cases (29%), HPV-18 in 4 of 21 (19%), HPV-6 in 3 of 21 (14%), HPV-16 in 3 of 21 (14%), HPV-33 in 2 of 21 (10%), HPV-30 in 1 of 21 (5%), plus two isolates (CP8304 and IS324).

Discussion

Genital HPV are commonly detected from clinical samples by consensus PCR methods (Gravitt et al, 2000). Two commonly used primer systems, the MY09/11 primers and the GP5+/6+ primers, amplify a broad spectrum of HPV genotypes (de Roda Husman et al, 1995; Resnick et al, 1990). A number of other consensus primers specific for the L1, E6, E6/E7, E7/E1, and E1 HPV regions have been described, each of which allows for the detection of a wide spectrum of HPV genotypes (Poljak et al, 1998). In our assay, the GP5+/6+ and MY09/11 primer sets were used for amplification of clinical specimens by PCR. However, depending on the purpose of the task, other consensus primer sets or type-specific primers could be applied for pyrosequencing.

Table 1. Pyrosequencing HPV Genotyping Results Compared with Results from Conventional DNA Sequencing and Type-Specific PCR

Number of samples	Number of bases needed for HPV genotyping by pyrosequencing	HPV typing by pyrosequencing	HPV typing by conventional DNA sequencing (ABI 310)	HPV typing by type-specific PCR
4	14	HPV-6	HPV-6	HPV-6
26	17	HPV-16	HPV-16	HPV-16
12	17	HPV-18	HPV-18	HPV-18
1	18	HPV-30	HPV-30	HPV-30
12	21	HPV-31	HPV-31	HPV-31
5	15	HPV-33	HPV-33	HPV-33
1	18	HPV-35	HPV-35	HPV-35
1	19	HPV-45	HPV-45	HPV-45
1	15	HPV-52	HPV-52	HPV-31[a]
1	18	HPV-59	HPV-59	HPV-59
1	18	HPV-66	HPV-66	HPV-66
1	18	CP8304	CP8304	CP8304
1	38	IS324	IS324	IS324

HPV, human papillomavirus.

[a] The sample that was mistyped by type-specific PCR as HPV-31 was typed correctly as HPV-52 by pyrosequencing and conventional DNA sequencing.

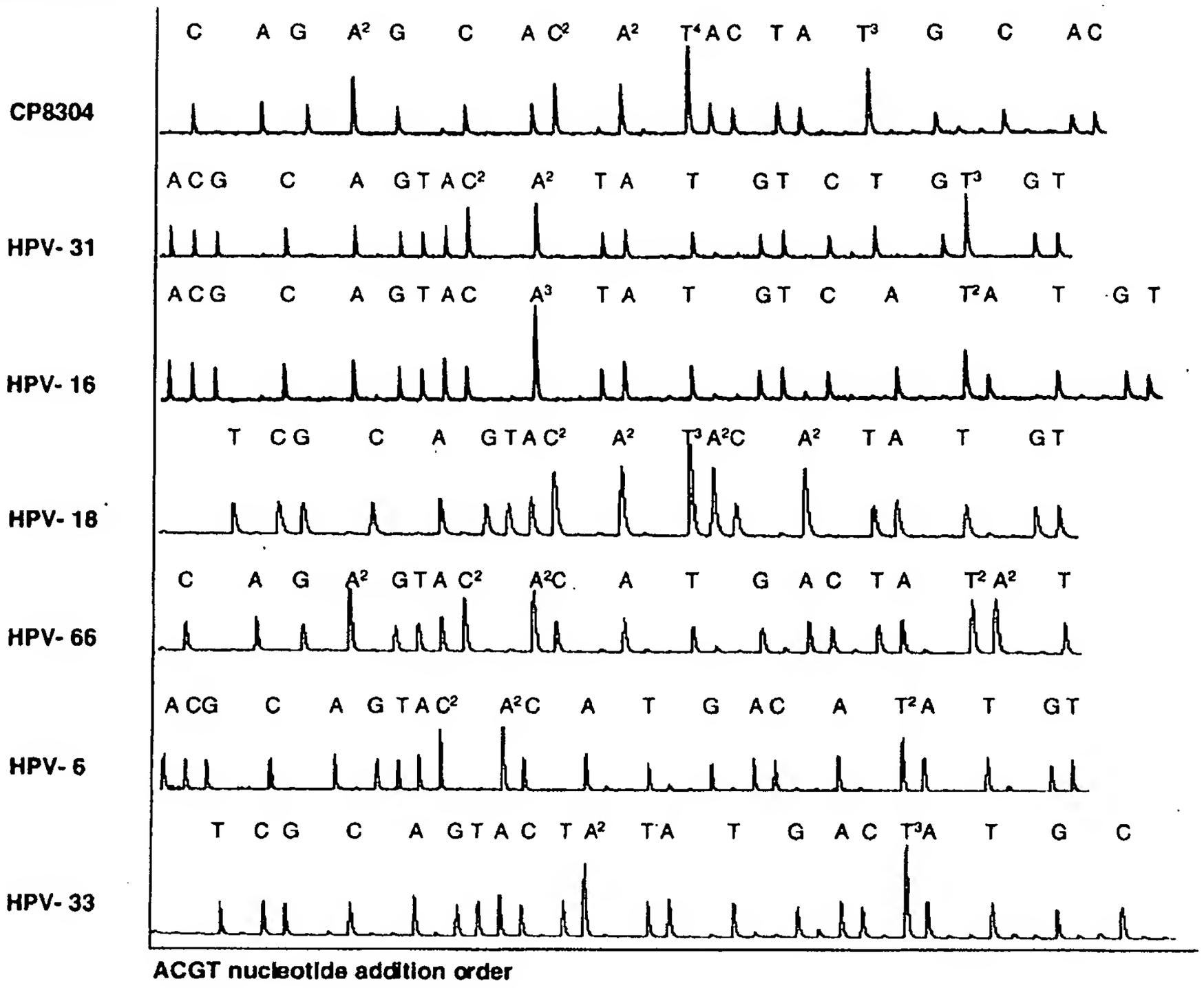

Figure 3.
Raw pyrogram sequence data from seven HPV amplicons amplified with GP5+/6+ primers.

Here we report, for the first time, the use of pyrosequencing, a novel DNA sequencing method, for detection and genotyping of HPV. The objective was to sequence and genotype HPV samples with pyrosequencing, and compare the results with type-specific PCR method and conventional gel-electrophoretic DNA sequencing.

The sequences obtained by pyrosequencing and conventional DNA sequencing were in complete agreement, indicating the ability of the system to type HPV samples with high accuracy and throughput. Between 14 and 21 bases generally were required to genotype our samples, with the exception of one case, where 38 sequence bases were required (the sample was revealed at 18 bases as HPV). Longer sequencing (up to 50 bases) could be performed by pyrosequencing to cover all of the genotypes. Table 1 demonstrates the number of sequence bases needed for genotyping of each type that was sequenced by pyrosequencing.

The sample that had been earlier (mis)typed as HPV-31 by primer specific PCR was correctly genotyped as HPV-52 by both pyrosequencing and conventional DNA sequencing. This indicates that pyrosequencing is a highly precise tool for HPV genotyping.

Pyrosequencing is suitable also for mutation detection in HPV sequence variants harboring mutation(s); eg, a single T to C substitution was detected in HPV-31 (Fig. 4).

Furthermore, we performed PCR on cell lysates to investigate the possibility of one-step amplification directly from cytobrush cell specimens. Multiple bands were visible after gel staining from most of the amplicons. Only 4 of 12 samples had a single band after gel staining, giving a low signal intensity in pyrosequencing. The sequence data obtained from the remaining amplicons suggests the existence of unspecific amplification along with a low amount of amplified DNA. Unspecific amplifications with the consensus primer set My09/11 has previously been described (Fernandez-Contreras et al, 2000). The cell lysate samples were amplified using nested PCR, which yielded remarkably high signal intensities, characteristic of high amounts and specific PCR products. Thus, nested PCR is recommended for direct amplifi-

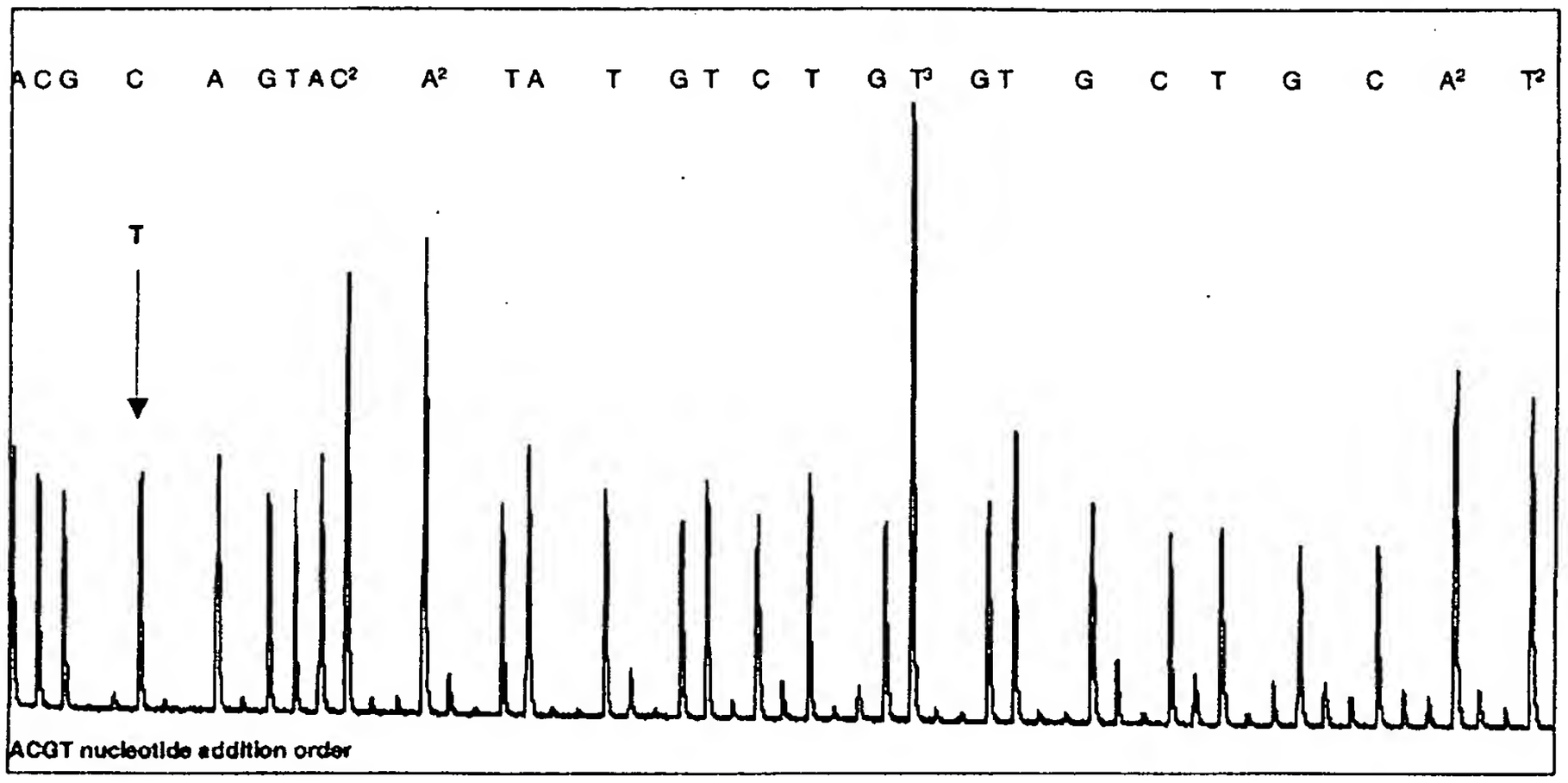

Figure 4.
Pyrogram for HPV-31. A single base T to C substitution was detected by pyrosequencing.

cation from cell lysates or in cases of unspecific amplification from DNA extracts. To maintain the performance characteristics (the sensitivity and specificity of the system) in pyrosequencing, samples should be sequenced when a clear and specific product band is seen on ethidium bromide-stained agarose gels after PCR amplification.

Use of pyrosequencing as a tool for HPV genotyping offers a relatively simple technology that eliminates the misclassification in broad-spectrum HPV genotypes that can occur with nonsequencing methods (Poljak et al, 1998). Additionally, pyrosequencing was found to be a reliable technique to identify novel HPV types. In hybridization methods, these novel types either are not detected or are misclassified because of cross-hybridization with a closely related type (Vernon et al, 2000).

As with other available methods, multiple infections present in one specimen might be problematic to detect, depending on the proportional dominance and number of genotypes present in the amplicon. At present, pyrosequencing might not be particularly useful for identifying infection with more than one HPV genotype, because multiple infections give sequence signals from all of the available types in the specimen. Typing may be possible, provided one type is solidly dominant, with low background signal(s) from other existing genotype(s). However, this information alone may be insufficient. For example, it would be problematic to identify the presence of a low-risk type of HPV, but fail to identify the presence of a (subdominant) high-risk HPV in a mixed infection. A possible solution to this issue is to use high-risk HPV-specific sequencing primers for sequencing.

In conclusion, pyrosequencing is a rapid, reliable, and robust system for detection of HPV. It is appropriate for routine clinical screening with large numbers of samples, and is easily adapted to laboratory automation. Pyrosequencing also enables detection of a broader spectrum of HPV, including putative novel types and mutations.

Materials and Methods

HPV Samples

Sixty-seven cervical samples were used in our test. Thirty-five samples were from cervical cancer patients, 21 from screened healthy individuals, and 11 from dysplastic individuals. Fifty-five of the samples were amplified from DNA extract (Hagmar et al, 1995) and the remaining 12 samples were amplified directly from cell lysates. All dysplasia and cancer samples were fresh-frozen biopsies, whereas the normal (screening) samples were cellular samples (cytobrush). Extraction was performed with phenol/chloroform followed by ethanol precipitation.

HPV PCR

The DNA amplifications were performed in 50 μl mixtures consisting of 5 μl of DNA sample, 5 μl of PCR buffer (Perkin-Elmer, Norwalk, Connecticut), 3.5 mM $MgCl_2$, 0.2 mM dNTP, 25 pmol of GP5+/6+ (de Roda Husman et al, 1995; Snijders et al, 1990) primer set, and 1 U of AmpliTaq (Perkin Elmer). The GP6+ primer was biotinylated. The thermocycler temperature program consisted of denaturation at 94° C for 1 minute, annealing at 38° C for 1 minute, and extension at 71° C for 2 minutes during 40 cycles. Each PCR was initiated with a 4-minute denaturation step at 94° C and finished by a 4-minute extension step at 71° C. A Perkin-Elmer 9700 thermocycler was used for all amplifications.

HPV Nested PCR

Cervical cells from 12 cytobrush samples were suspended in 1 ml of 10 mM Tris-HCl (pH 7.4). After one-time freezing and thawing, the samples were boiled for 10 minutes at 100° C. DNA from cervical cell lysates was amplified by two sets of general primers within the L1 open-reading frame. The MY09/11 consensus primer set was used in combination with the GP5+/6+ general primer set in a nested, two-step amplification. The DNA amplifications were performed in 50 μl mixtures containing 5 μl of prepared DNA sample from the cell lysate, 5 μl of PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 2 pmol of primer MY09/11, and 1 U of AmpliTaq. The thermocycler temperature program consisted of denaturation at 95° C for 30 seconds, annealing at 45° C for 30 seconds, and extension at 72° C for 1 minute during 35 cycles. Each PCR was initiated by a 5-minute denaturation step at 95° C and finished by a 10-minute extension step at 72° C. Five microliters of amplified DNA was used as the template for the second PCR with the GP5+/6+ primer pair. The procedure for the second PCR was as described above for HPV PCR.

Single-Strand Template Preparation for Pyrosequencing

Fifty microliters of biotinylated PCR product was immobilized onto 200 μg of streptavidin-coated super paramagnetic beads (Dynabeads M-280–streptavidin, Dynal AS, Oslo, Norway) by incubation at 43° C for 30 minutes. Single-stranded DNA was obtained by incubating the immobilized PCR product in 5 μl of 0.1 M NaOH for 4 minutes. The immobilized strand was suspended in 8 μl of H_2O plus 1 μl of annealing buffer (100 mM Tris-acetate pH 7.75, 20 mM Mg-acetate). Single-stranded DNA corresponding to 50 μl of PCR product was hybridized to 10 pmol of GP5+ sequencing primer at 70° C for 3 minutes, and incubated at room temperature for 5 minutes.

Pyrosequencing

The primed PCR product was added to the pyrosequencing reaction mixture containing 0.1 M Tris-acetate pH 7.75, 0.05% Tween 20, 10 U of exonuclease deficient (exo$^-$) Klenow DNA polymerase, 50 mU of apyrase (Sigma, St. Louis, Missouri), 0.8 μg of purified luciferase (BioThema, Dalarö, Sweden), 15 mU of recombinant ATP sulfurylase (Karamohamed et al, 1999), 0.5 μg of single-stranded DNA-binding protein (Amersham Pharmacia Biotech, Uppsala, Sweden), 0.5 mM EDTA, 5 mM Mg-acetate, 0.1% bovine serum albumin (BioThema), 1 mM dithiothreitol, 5 μM adenosine 5'-phosphosulfate (Sigma), 0.4 mg/ml of polyvinylpyrrolidone (360 000), and 100 μg/ml of D-luciferin (BioThema) in a total volume of 50 μl. Pyrosequencing was performed at 28° C on an automated pyrosequencer PSQ 96 (Pyrosequencing AB, Uppsala, Sweden; www.pyrosequencing.com) at a dispensing pressure of 600 mbar with 8-msec open times and 65-second cycle times. The sequencing procedure was carried out by stepwise elongation of the primer strand upon cyclic dispensation of the different deoxynucleoside triphosphates (Amersham Pharmacia Biotech). A CCD camera detected the light output resulting from nucleotide incorporation. The data were obtained in Microsoft Excel and graphic format.

Conventional DNA Sequencing

The sequencing data obtained from pyrosequencing were confirmed by DNA sequencing on an ABI 310 (Perkin Elmer), using BigDye terminator chemistry, as described previously (Elfgren et al, 2000).

Type-Specific PCR

The samples were genotyped by the type-specific PCR method. The consensus PCR procedures with My09/11 general primers and the type-specific PCR detection were previously described in detail (Hagmar et al, 1995; Skyldberg et al, 1991).

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Molecular detection and genotyping of human papillomavirus

Leen-Jan van Doorn†, Berhard Kleter and Wim GV Quint

Human papillomavirus infections are associated with the development of cervical neoplasia. Human papillomavirus is a group of heterogeneous viruses, comprising many genotypes, which can be divided into high-risk and low-risk types, depending on their association with disease. Therefore, accurate molecular diagnostic tools are required for detection and identification of human papillomavirus. Monitoring of human papillomavirus infection is necessary for adequate patient management and follow-up during treatment. This review describes the different molecular methods available for human papillomavirus detection and identification of genotypes.

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Human papillomaviruses (HPV) infect epithelial cells of the skin and mucous membranes in humans. Infection of cutaneous epithelia can cause warts, whereas infection of the anogenital region can cause genital warts as well as various forms of cancer both in men and women. Cervical cancer is one of the most common forms of cancer in women worldwide, only second to breast cancer. The main interest in HPV is due to its causative role in cervical cancer. Worldwide, approximately 400,000 new cases of cervical cancer are diagnosed annually and more than 200,000 women die of this disease, 80% of which occur in developing countries [1,2].

The Papanicolaou (Pap) cervical smear has been considered the standard method to detect cytologic abnormalities in cervical epithelium by microscopic examination. Recently, liquid cytology media have been introduced, facilitating cytologic classification [3]. Over past decades, classification systems for cervical smears and biopsy specimens have evolved from the original Pap classification towards the cervical intraepithelial neoplasia (CIN) grading system [4] and the Bethesda system [5]. To classify abnormal specimens, the latter system comprises atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesion (HSIL) [6].

The HPV virion is approximately 55 nm in diameter and the outer coat comprises major and minor capsid proteins. HPV has a double-stranded circular DNA genome of approximately 7900 bp, with early (E) and late (L) genes and an untranslated control region (FIGURE 1). The L1 and L2 genes encode the major and minor capsid proteins. The E genes regulate viral replication and in some cases have transformation potential [7,8].

At present, more than 100 different HPV types have been identified based on differences in DNA sequence. These HPV types can be classified by various criteria, e.g., their tissue tropism, oncogenic potential and phylogenetic classification. Based on their biological niche, mucosal (anogenital and oral) and cutaneous (skin) HPV types can be recognized. Among the HPV types infecting the anogenital epithelia, high-risk and low-risk genotypes are defined, depending on their presence in cervical carcinoma or precursor lesions [9]. For example, HPV types 6 and 11 are considered as low-risk genotypes, whereas HPV 16 and 18 are classified as high-risk genotypes. The classification as low- or high-risk is still under debate for several HPV types, mainly due to their low overall prevalence. Since new HPV types are still being discovered, the spectrum of HPV genotypes is not yet complete.

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Multiple sequence alignments of complete or partial HPV genomes showed a high degree of genetic heterogeneity among HPV isolates. Phylogenetic analyses have revealed the existence of several clades of HPV genotypes and in general, these correlate with tissue tropism and oncogenic capacity. Since recombination between different HPV genotypes appears to be very rare, genotypes can be reliably classified by analysis of only part of the viral genome [10]. The choice of the genomic region used for typing of viral isolates is crucial. This region has to show sufficient discriminatory power, i.e., intertypic variation, to distinguish all the different genotypes. The intratypic variation, i.e., the heterogeneity among isolates belonging to the same genotype, should be limited to permit reliable identification. Since biological characterization of HPV is limited by the lack of *in vitro* culture systems and reliable serological reagents, classification of HPV is almost entirely based on molecular characteristics. There is general agreement about the nomenclature of HPV genotypes and the general classification of HPV in the field but this has not yet been officially accepted by the International Committee on Taxonomy of Viruses and is still under debate.

Diagnosis of HPV infections

HPV cannot be adequately cultured *in vitro* and serological assays only have limited accuracy, since they cannot distinguish between current and past infection [11]. Therefore, diagnosis of HPV relies entirely on the detection of the viral DNA in clinical samples.

Cytologic and histologic examination does not permit direct assessment of HPV itself but diagnoses the consequences of viral infection. Therefore, detection of HPV-DNA can provide more sensitive diagnosis of infection and the associated risk for development of cervical neoplasia.

Numerous studies have investigated the prevalence of HPV in various populations worldwide and showed a wide range of HPV-DNA positivity rates [12–15]. In general, the prevalence of HPV is higher in younger women as compared with women over 30 years with normal cytology [13,16,17]. This indicates that HPV infections are common in young women and that most of these resolve spontaneously. Most young women with a positive HPV test will become negative within a 24-month period [18].

These variable outcomes of HPV testing may be due to several important factors. First, there are remarkable differences among populations with respect to age, prevalence of cytologic abnormalities and presence of different HPV genotypes. Secondly, multiple sampling and HPV-DNA detection techniques have been used with different sensitivity and specificity and these may have a significant impact on the detection rate of HPV. At present, there are no international quality control panels available to compare the various diagnostic methods.

The natural history of HPV infection, including mode of transmission of the virus, development of persistent infection, clearance of the virus and the interaction with the immune system, is only partially known. At present, there is no established definition of a persistent HPV infection. A recent study suggested that women with mild or moderate dyskaryosis should only be referred for treatment after a persistent HPV infection of at least 6 months [19]. However, detection of HPV-DNA in consecutive samples should include genotyping or even analysis of molecular variants to confirm persistence of the same virus over time [20]. Since the prevalence and persistence of HPV infection is much higher in immunocompromised (such as HIV-infected) women, the role of the immune system is crucial for the course of an HPV infection [21,22].

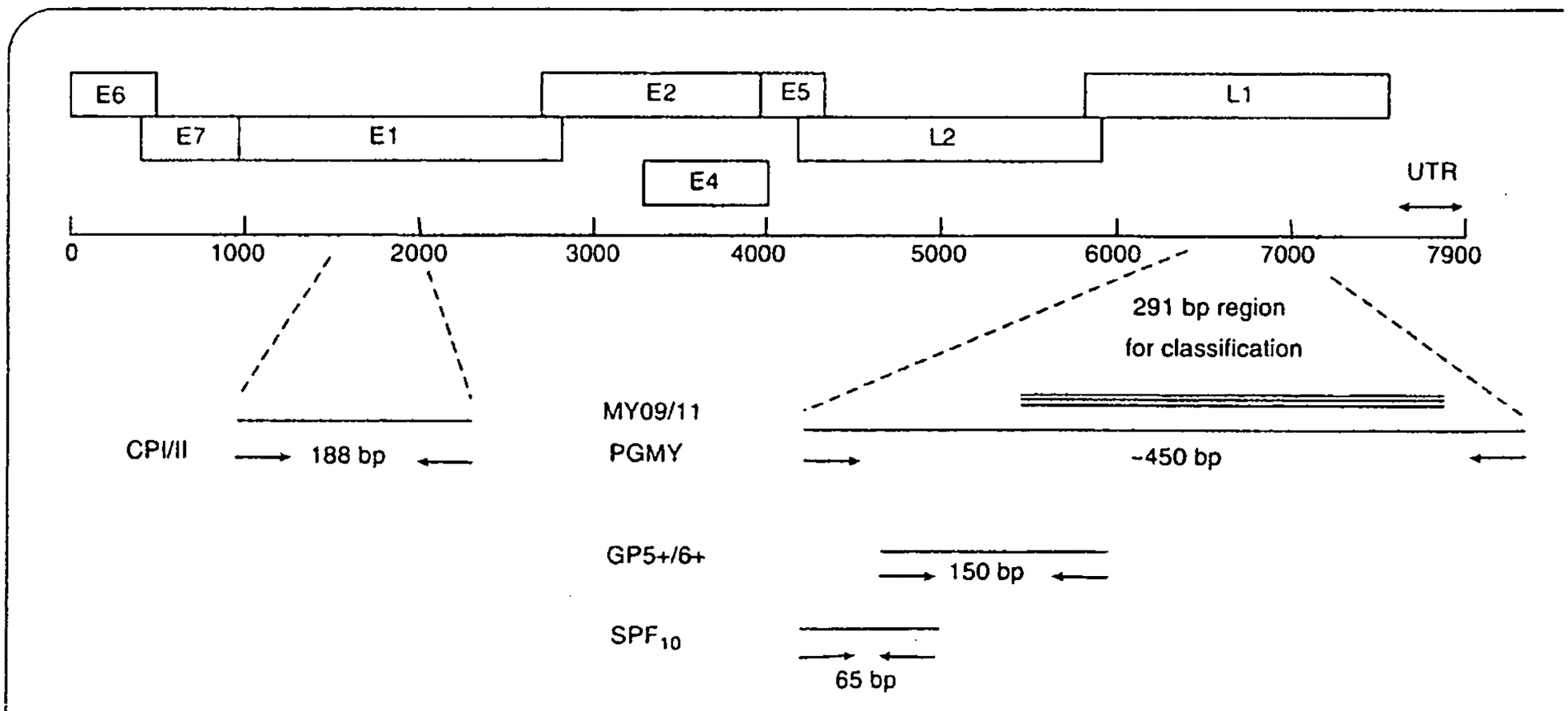

Figure 1. Outline of the HPV-DNA genome, presented in a linear form. The position of the early (E), late (L) genes and the untranslated region (UTR) is indicated, as well as the positions of the four most widely used general primer sets CPI/II [42], MY09/11[38], GP5+/6+ [40] and SPF_{10} [41] with their respective amplimer sizes. The 291 bp fragment used for formal classification of HPV genotypes [10] is shown in the L1 region.

Taken together, diagnostic test results should be interpreted with care [23,24]. Novel algorithms are being developed, combining cytologic screening and HPV-DNA analysis, to optimize the positive and negative predictive value for development of disease [19,25,26].

This review will focus on the different strategies and methods used for molecular detection of HPV-DNA and identification of HPV genotypes.

Detection of HPV-DNA & identification of HPV genotypes

HPV can be detected in cervical smears and biopsy specimens by *in situ* hybridization. This method is based on the use of labeled probes that specifically hybridize to HPV-DNA leaving the morphology of the infected cells intact. This permits localization of HPV infection in the sample and possible colocalization with other markers [27–29]. Identification of HPV genotypes would require the use of type-specific probes in multiple *in situ* hybridization experiments. Alternatively, HPV-DNA can be directly isolated from clinical samples and detected by Southern blot or dot spot hybridization. However, this approach lacks the necessary sensitivity for adequate clinical application [30–32]. These systems also are labor-intensive and are not suitable for high-throughput screening studies. Therefore, nucleic acid amplification methods have been developed to increase the sensitivity as well as the specificity of HPV-DNA detection. In general, nucleic acid detection methods comprise three distinct steps:

- First, the nucleic acid is released from the clinical sample
- Secondly, the nucleic acid of interest is amplified
- Thirdly, the amplified material is analyzed to detect presence of HPV and identify HPV genotypes

Sample collection & nucleic acid isolation for HPV-DNA analysis

HPV-DNA assays can be performed using the same specimen collection medium as used for cytologic examination, which is an important logistic aspect for routine clinical testing. It should be noted that sampling errors can play an important role when using highly sensitive molecular assays. A cervical scrape is only a small sample of the cervical epithelium. These epithelial cells are then suspended in a certain volume of transport medium, of which only part is used for DNA isolation. A fraction of the isolated DNA is subsequently included in the PCR. Therefore, if a sample only contains a limited number of HPV-DNA copies even a sensitive assay may be hampered by sampling error. This not only has consequences for determining whether HPV-DNA is present or absent but also for genotyping of HPV-DNA-positive samples. Sampling errors are particularly important when multiple HPV genotypes are present at different concentrations. A first test could reveal two or three different genotypes, whereas repetition of the test on another aliquot reveals only one or two genotypes or *vice versa*. Therefore, a representative portion of the original clinical samples should be included in each assay. A recent study revealed that analysis of cervical scrapes as well as biopsy specimens from the same patient yielded highly comparable but not identical HPV genotyping results [33]. Thus, sampling errors should always be taken into account.

Stability of the sample during transport and storage is also important. The viral nucleic acid should be preserved to avoid false-negative results caused by degradation by endogenous endonucleases. This is especially important when analyzing HPV-RNA transcripts. To assess the integrity of the genomic DNA in the sample and the suitability for molecular analysis, adequate controls, such as β-globin gene amplification or spiking of the sample with a positive control, are a crucial part of each method.

A wide variety of methods are available to release HPV-DNA from the infected cells and to make it available for subsequent detection. These range from a simple protease treatment, yielding a crude lysate which is immediately used for amplification to multistep purification methods of nucleic acid isolation.

Signal amplification

The Hybrid Capture II system (HCII, Digene Corp., USA) is a nonradioactive signal amplification method, based on the hybridization of the target HPV-DNA to labeled RNA probes in solution [34,35]. The resulting RNA–DNA hybrids are captured onto microtiter wells and are detected by a specific monoclonal antibody and a chemiluminiscent substrate, providing a quantitative measurement of HPV-DNA. Two different probe cocktails are used, one comprising probes for low-risk genotypes 6, 11, 42, 43 and 44; and the other containing probes for high-risk genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. This assay has become the standard in many countries, is widely used in clinical studies and has been FDA approved. However, the HCII test has several disadvantages. The detection limit of the HCII is approximately 5000 genome equivalents and thus is less sensitive than PCR [36,37]. The HCII assay does not permit identification of specific HPV genotypes but only distinguishes between the high-risk and low-risk groups. The accuracy of the test is hampered by cross-reactivity of the two probe cocktails. Since determination of HPV persistence requires the identification of the specific HPV genotype, the HCII test cannot be used for such studies.

Target amplification

PCR is the most widely used target amplification method, employing oligonucleotide primers flanking the region of interest, which is amplified by a thermostable DNA polymerase during a thermocycling process. For detection of HPV-DNA, two different PCR approaches are possible.

The first method employs type-specific PCR primers, which are designed to exclusively amplify a single HPV genotype. Thus, to detect the presence of HPV-DNA in a single clinical sample, multiple type-specific PCR reactions should be performed separately. This method is labor-intensive and expensive and the type-specificity of each PCR primer set should be validated.

The second method is based on the use of consensus or general PCR primers to amplify a broad-spectrum of HPV genotypes. PCR primers are aimed at conserved regions among the different HPV genotypes. Identification of such conserved regions requires complex alignments of multiple sequences from each HPV genotype. Since the L1 region is the most conserved part of the genome, several popular consensus PCR primer sets are aimed at this region [38–41]. General primers also have been described in the E1 region (FIGURE 1) [42]. Apart from these most frequently used PCR primer sets, several other broad-spectrum PCR primers have been reported [43–46].

Three strategies for broad-spectrum HPV-DNA amplification

There are three different designs of general PCR primer sets that can be used to achieve broad-spectrum detection of HPV-DNA.

The first design chooses one forward and one reverse oligonucleotide primer aimed at a conserved genomic region but fully matching only one or a few HPV genotypes. To compensate for the mismatches with other HPV genotypes, the PCR is performed at a low annealing temperature. The GP5+/6+ PCR system is an example of this approach [40].

The second class of general PCR primer sets comprises of forward and reverse primers, which contain one or more degeneracies to compensate for the intertypic sequence variation at the priming sites. These primers do not have to be used at a lower annealing temperature. The My11/09 is an example of a degenerated PCR primer set [38]. In fact, this primer set comprises a mixture of many different oligonucleotides. The disadvantage of this design is that synthesis of oligonucleotides containing degeneracies is not very reproducible and results in high batch-to-batch variation. Therefore, each novel batch of primers should be carefully evaluated to check the efficacy of amplification for each HPV genotype [39].

The third option for a broad-spectrum PCR primer set is to combine a number of distinct forward and reverse primers, aimed at the same position of the viral genome. These primers do not contain random degeneracies but may contain inosine, which matches with any nucleotide. Using a defined mixture of nondegenerate primers has the advantage that the oligonucleotides can be synthesized with high reproducibility and PCR is performed at optimal annealing temperatures. Examples of such primer sets are the PGMY primers [39] and the SPF_{10} primers [41,47].

Besides the choice of primers, the size of the PCR product is important. In general, the efficacy of a PCR decreases with increasing amplimer sizes. Some clinical samples have been subjected to treatments that damage the DNA, such as formalin-fixation and paraffin-embedding. Due to this DNA degradation, the efficacy of PCR primers generating a small product, such as the SPF_{10} is considerably higher as compared to primer sets yielding larger amplimers [41].

Real-time PCR can also be used to detect HPV-DNA. Type-specific PCR primers are combined with type-specific fluorescent probes for real-time detection [48–50]. However, broad-spectrum PCR primers are less suitable for real-time PCR. PCR products can be detected by general fluorescent dyes, such as SYBR Green but the specificity is limited and can generate false-positive results. Due to the sequence heterogeneity of the different HPV genotypes, PCR products of broad-spectrum PCR will not be detected adequately with a single fluorescent probe but requires a mixture of probes. Since these probes will all have different characteristics, standardization is difficult [51]. For the same reason, quantification of HPV-DNA by real-time PCR is restricted to type-specific PCR primers.

Detection & analysis of amplification products

Amplimers can be easily detected by standard agarose gel electrophoresis. However, subsequent hybridization analysis considerably increases both the sensitivity and specificity of the assay. Type-specific PCR products can be confirmed with corresponding type-specific probes. However, HPV sequences amplified by broad-spectrum PCR primers comprise heterogeneous interprimer sequences and require multiple probes to detect all possible HPV genotypes amplified. Therefore, hybridization analysis of broad-spectrum PCR requires cocktails of probes, as used in the hybrid capture method. To increase the throughput of a diagnostic assay, hybridizations could be performed in microtiter plates [40,41,52].

After PCR amplification, the HPV genotype is deduced from the amplified sequence by various methods.

Direct sequence analysis of PCR products

PCR products can be directly sequenced to assess the sequence between the PCR primers. Rapid sequencing methods are now becoming available for high-throughput to permit application in routine analysis of clinical samples [53] provided that adequate postsequencing analysis is available. However, it should be noted that sequence analysis is not very sensitive to simultaneously detect different sequences in a mixture. Sequences only representing a minority of the total PCR product may easily remain unnoticed and only the predominant genotype will be detected. This may be insufficient to analyze clinical samples containing a mixture of different HPV genotypes and will underestimate the prevalence of infections with multiple HPV genotypes, which has important consequences, for example, in vaccination or follow-up studies [47]. One of our recent studies compared direct sequence analysis of SPF_{10} PCR products with reverse hybridization in 166 HPV-positive cervical scrapes. HPV genotypes were compatible in all samples but direct sequence analysis found multiple types in only 2% of the cases; while reverse hybridization by the SPF_{10} LiPA found multiple types in 25% of the samples. Thus, direct sequencing is not a preferred method for accurate determination of HPV genotypes in clinical samples. The presence of multiple HPV genotypes is a common phenomenon in some patient groups. Up to 35% of HPV-positive samples from patients with advanced cytologic disorders and more than 50% of HIV-infected patients [54] contain multiple HPV genotypes, whereas multiple genotypes in carcinoma patients are less prevalent [47].

When a HPV sequence is obtained, the genotype can be deduced by two methods. First, the sequence can be used as a query for a homology search to sequences in a database. At present, extensive databases are available on the internet and can be accessed for free (for example [101]) using the BLAST software [55]. This tool permits fast homology searches of a sequence with all sequences in a combined and continuously updated sequence database. The result of such a homology search is a list of sequences in the database that show the highest homology with the query sequence.

Secondly, phylogenetic analyses can be performed. The novel sequence can be used in a multisequence alignment with a set of known HPV sequences, representative of different HPV genotypes. Based on the sequence alignment, a phylogenetic tree can be constructed, providing a graphical representation of the evolutionary relationships between the novel sequence and reference sequences.

Classification of genotypes is entirely based on sequence analysis of the viral genome. By definition, HPV isolates belong to different types if a defined 291 bp part of the L1 open reading frame (FIGURE 1) differs at least 10% [10]. The nomenclature is organized and maintained by an international committee to ensure consensus in the scientific community.

PCR & restriction fragment length polymorphism

After amplification, the sequence composition of a PCR product can be investigated by restriction enzymes. Digestion of PCR products with various restriction endonucleases generates a number of fragments, which can be resolved by gel electrophoresis yielding a particular banding pattern. If mutations occur at the restriction site, the endonuclease will not recognize the site anymore and will fail to digest the DNA, resulting in different restriction fragments changing the original banding pattern. This method is relatively easy but is labor-intensive and requires adequate quality control of the restriction enzyme. More importantly, the application of this method depends on the availability of suitable restriction enzymes to permit detection of specific mutations in the target sequence. Detection of multiple HPV genotypes present in different quantities in a clinical sample by PCR and restriction fragment length polymorphism (PCR-RFLP) is usually complex and the sensitivity to detect minority genotypes is limited [55,56].

Hybridization analysis of PCR products

The most common way to investigate the sequence of PCR products is hybridization with one or more oligonucleotide probes. The best known method is Southern blotting, where the HPV-derived PCR product is electrophoresed on a gel, transferred to a membrane and subsequently hybridized to a labeled probe [31]. However, Southern blotting is labor-intensive and not suitable for routine application and high-throughput analyses. Therefore, alternative hybridization formats have been developed.

Microtiter hybridization assay

Hybridization to oligonucleotide probes can be performed in a microtiter format, as shown in FIGURE 2 [41]. PCR products are generated whereby one of the primers is labeled with biotin. After amplification, PCR products are captured onto streptavidin-coated microtiter wells. Double-stranded DNA is denatured under alkaline conditions and the uncaptured strand is removed by washing. A labeled oligonucleotide probe is added, which can hybridize to the captured strand. After stringent washing, hybrids can be detected by conjugate and substrate. An advantage of this method is the high-throughput of the microtiter format and the availability of automates and equipment to reduce the hands-on time. Therefore, it is suitable to distinguish between HPV-DNA-positive and -negative samples as a first step in HPV molecular diagnosis. However, identification of each individual HPV genotype by a specific probe requires hybridization in a separate well and a large volume of PCR product is needed [40].

Reverse hybridization methods

To permit simultaneous hybridization of a PCR product to multiple oligonucleotide probes, reverse hybridization offers an attractive tool. This method comprises immobilization of multiple oligonucleotide probes on a solid phase and providing the PCR product in the liquid phase. Hybridization is performed in a single step and hybrids are detected by different methods.

The best known reverse hybridization technology comprises a membrane strip containing multiple probes immobilized as parallel lines (line probe assay; LiPA). A PCR product is generated,

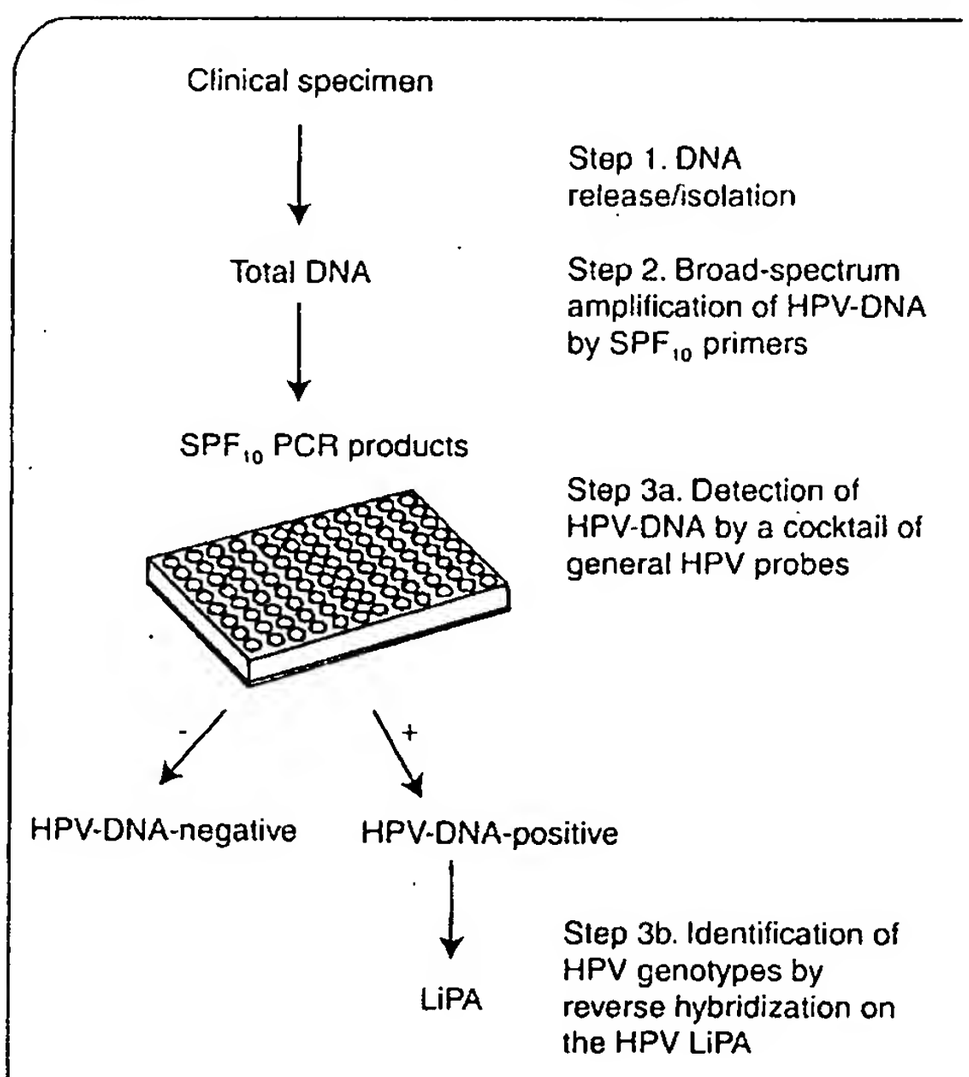

Figure 2. Three-step molecular detection of HPV-DNA. Total DNA is isolated from a clinical specimen and amplified by the SPF_{10} PCR primer set. HPV-DNA-positive samples are detected by a microtiter format general hybridization assay and HPV genotypes can be subsequently identified using the same PCR product on a line probe assay (LiPA).

using biotinylated primers. The double-stranded PCR product is denatured under alkaline conditions and added to the strip in an appropriate hybridization buffer. After hybridization and stringent washing, the hybrids can be detected by addition of a streptavidin-conjugate and a substrate, generating a purple precipitate at the probe line. Hybridization patterns can be visually interpreted. This method permits multiparameter testing in a single step and requires only a limited amount of PCR product. The reverse hybridization line probe assay technology has been used to diagnose and genotype various microorganisms, such as hepatitis C virus [58], human immunodeficiency virus [59] and *Helicobacter pylori* [60,61]. An example of the HPV LiPA [33,47,62,63] is shown in FIGURE 3.

Alternative reverse hybridization methods for HPV genotyping are the line blot assay [64–68] and the HPV-DNA chip (SK Global, Seoul, Korea). All currently available reverse hybridization methods are subject to further evaluation studies to establish their role in clinical and epidemiological studies.

Expert opinion & five-year view

The development of highly sensitive DNA detection assays over the past years has revolutionized the diagnosis of HPV. Now that novel methods are available, they can be used to study various crucial aspects of HPV infections.

The implications of HPV-DNA detection for patient management must be further developed. Recent studies have shown that the prevalence of HPV-DNA positivity and the prevalence of multiple HPV genotypes in the same patient is higher than assumed. Furthermore, the efficacy of large community-based HPV screening studies depends on the accuracy of the diagnostic assays used. To identify women with an increased risk for cervical neoplasia, it is obvious that detection of HPV-DNA alone is insufficient and additional aspects need to be included in the diagnosis. Accurate genotyping is essential for adequate classification of patients in a low-risk or high-risk group. Furthermore, there is preliminary evidence that presence of multiple HPV genotypes may reflect repeated exposure and may also be related to an increased risk for development of disease. HPV persistence also has been identified as an important risk factor. However, HPV infections can only be classified as persistent if identical (sub)types are identified in consecutive samples during follow-up studies, which is not possible by current HPV screening methods. The viral load appears to be a valuable marker for prediction of disease. Since general quantitative assays (which are sufficiently sensitive) are difficult to develop, genotyping will also play an important role for selection of the right type-specific quantitative assay. Finally, the development of type-specific antiviral therapies or vaccines requires the implementation of adequate algorithms for detection and genotyping of HPV. These methods are necessary for accurate follow-up during clinical trials, monitoring of (antiviral or surgical) treatment as well as triage and management of patients during community-based screening studies.

To address cervical cancer worldwide, it is important to perform extensive epidemiological studies to assess the geographic distribution of HPV genotypes. Given the extensive

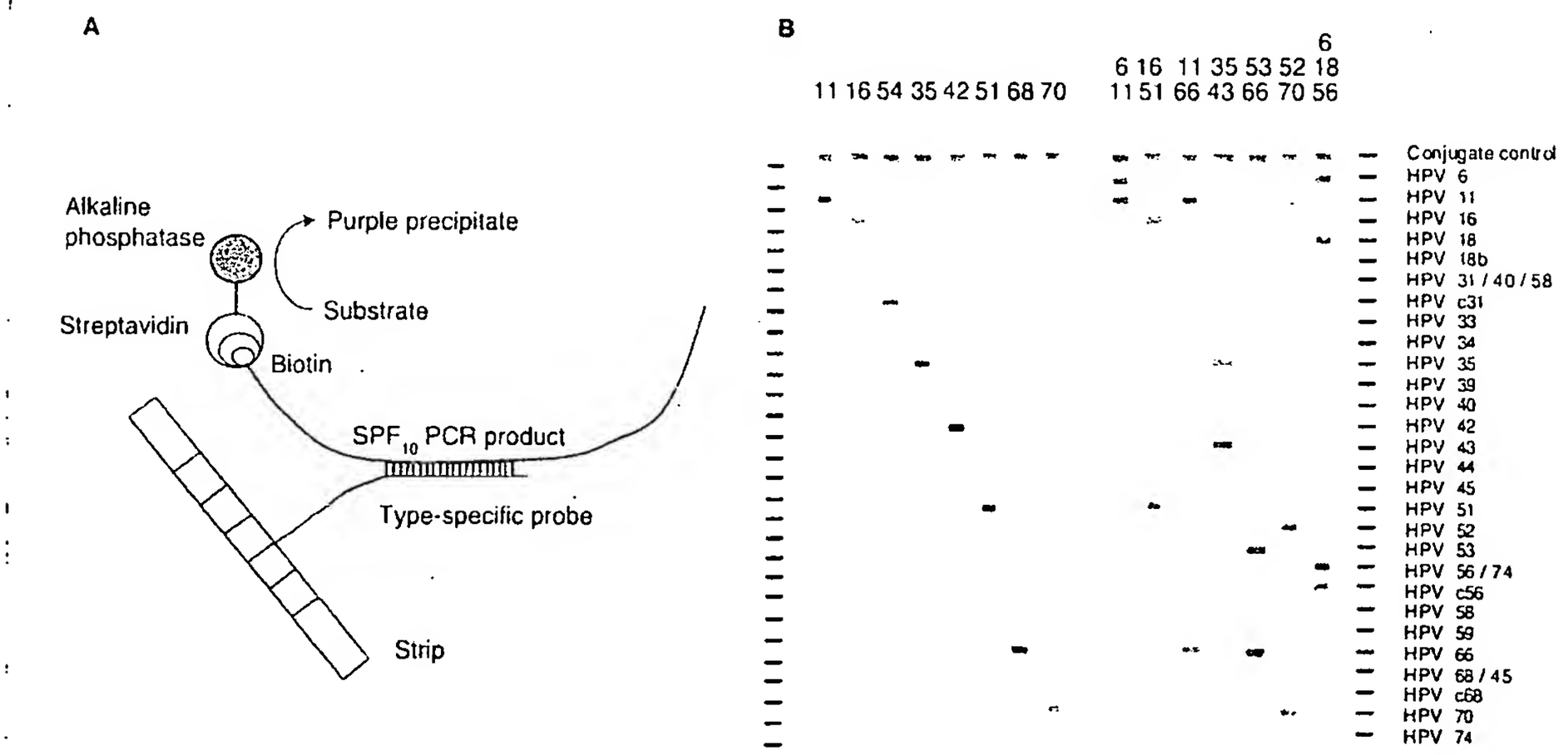

Figure 3. Outline and example of the reverse hybridization HPV line probe assay (LiPA).
A. Amplimers are denatured and hybridized under stringent conditions to probes that have been immobilized as parallel lines on a strip. After stringent washing, the hybrids are detected by alkaline-phosphatase conjugated streptavidin and a substrate, yielding a purple precipitate.
B. Examples of specimens containing single (left) or multiple (right) HPV genotypes. The HPV genotypes can be deduced by visual interpretation of the hybridization patterns after alignment with the probe line template as shown on the right side.

genetic heterogeneity of HPV, specific molecular tools will be required. Besides the identification of high-risk genotypes, also further detection of specific subtypes may be clinically relevant. Therefore, novel low- or high-density DNA probe arrays (DNA chips) may provide a useful technology for identification of HPV genotypes and subtypes in clinical and epidemiological studies.

Information resources

Apart from numerous websites providing information for the general public, one of the most informative sites for molecular data is at http://hpv-web.lanl.gov, at the Los Alamos laboratories. A compendium of information called 'Human papillomavirus: a compilation and analysis of nucleic acid and amino acid sequences' is available at this site, including all HPV genotypes and several subtypes.

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Key issues

- Implement the use of highly sensitive HPV-DNA assays in clinical studies.
- Implement the use of sophisticated, standardized and evaluated HPV genotyping assays in clinical studies.
- Determine the geographic distribution of HPV genotypes worldwide in preparation of vaccine trials.
- Extend studies on the natural history of HPV infections and the role of the humoral and cellular immune responses.
- Introduce HPV genotyping and quantitative HPV-DNA testing in clinical follow-up studies to assess persistent infection.
- Establish international quality control programs for monitoring proficiency of different laboratories and preparation of quality control panels.

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Cervical human papillomavirus infection and squamous intraepithelial lesions in rural Gambia, West Africa: viral sequence analysis and epidemiology

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The development of effective strategies against cervical cancer in Africa requires accurate type specific data on human papillomavirus (HPV) prevalence, including determination of DNA sequences in order to maximise local vaccine efficacy. We have investigated cervical HPV infection and squamous intraepithelial lesions (SIL) in an unselected cohort of 1061 women in a rural Gambian community. Squamous intraepithelial lesions was diagnosed using cytology and histology, HPV was typed by PCR-ELISA of DNA extracts, which were also DNA sequenced. The prevalence of cervical HPV infection was 13% and SIL were observed in 7% of subjects. Human papillomavirus-16 was most prevalent and most strongly associated with SIL. Also common were HPV-18, -33, -58 and, notably, -35. Human papillomavirus DNA sequencing revealed HPV-16 samples to be exclusively African type 1 (Af1). Subjects of the Wolof ethnic group had a lower prevalence of HPV infection while subjects aged 25–44 years had a higher prevalence of cervical precancer than older or younger subjects. This first report of HPV prevalence in an unselected, unscreened rural population confirms high rates of SIL and HPV infection in West Africa. This study has implications for the vaccination of Gambian and other African populations in the prevention of cervical cancer.
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It is well established, on a cellular and epidemiological basis, that the sexual transmission of high oncogenic risk (HR) human papillomavirus (HPV) types is the essential prerequisite for cervical carcinogenesis (zur Hausen, 1977; IARC_Monographs, 1995; Muñoz and Bosch, 1996; Walboomers *et al*, 1999). Cervical cancer is the most common malignancy in West African women (Koulibaly *et al*, 1997; Bah *et al*, 2001) but accurate, population-based HPV prevalence data are lacking. Many therapeutic and prophylactic vaccines against HPV infection are in development, with some now entering phase III trials (Bosch *et al*, 2001; Nardelli-Haefliger *et al*, 2003; Taira, 2004; Tomson *et al*, 2004; Christensen, 2005; Gravitt and Shah, 2005; Maclean *et al*, 2005). Such vaccines are essential in developing countries where 80% of new cervical cancer cases occur but where screening and treatment of precancerous lesions is not available (Kitchener and Symonds, 1999; Lazcano-Ponce *et al*, 1999).

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Risk factors in the spread of genital HPV infection vary between populations, and include genetic variation in human leukocyte antigen (HLA) types resulting in differential susceptibility to HPV infection and socio-demographic factors such as sexual behaviour or age (Burk *et al*, 1996; Koutsky, 1997; Svare *et al*, 1998; Herrero, 1999). Consequently, prevalence of cervical cancer in industrialised nations cannot be assumed to apply in the developing world; local characterisation of HPV patterns is essential. Human papillomavirus-16 is the most prevalent type in cervical cancers worldwide (Bosch *et al*, 1995b), although regional variation in HR HPV types does occur (Bosch *et al*, 1995a; Herrero *et al*, 2000; Castellsague *et al*, 2001; Mayaud *et al*, 2001; Xi *et al*, 2003). HPV DNA sequence must therefore be defined to ensure vaccine efficacy and prevent the selective emergence of rare, virulent variants. Here we describe such detailed HPV analysis in a rural Gambian community, presenting the largest point-prevalence study to date in a rural unselected sub-Saharan African population.

METHODS

Population

Samples were collected during a large reproductive morbidity survey in rural Gambia between January and July 1999 as described elsewhere (Walraven *et al*, 2001). Briefly, the MRC has

run a continuous demographic surveillance system in 40 villages surrounding the provincial town of Farafenni since 1981. All women aged 15–54 years in 20 of these villages were approached and invited to participate. The study was approved by the ethics committee of The Gambian Government/MRC Laboratories (SCC proposal 755). All work was conducted in accordance with the Helsinki Declaration of 1975 as revised in 1983. After obtaining informed consent. A total of 1348 women (72% of those eligible) were seen and examined in dedicated village clinics.

Sampling

As part of the full gynaecological assessment, all consenting subjects who did not have an intact hymen underwent a vaginal speculum examination (Figure 1). Cervical smears were taken with an Aylesbury spatula for cytology (Wolfendale *et al*, 1987) fixed, stored and transported to Cardiff. Human papillomavirus sampling was performed by inserting the brush-sampler into the cervical canal and rotating it through 360°. This brush was placed in transport medium immediately (Digene Corporation, Gaithersburg, MD, USA), stored at −70°C and frozen samples transported to Cardiff for analysis.

Cytology

Women with abnormalities were offered further assessment and treatment with Large Loop Excision of the Transformation Zone (LLETZ) or knife cone biopsy. Histology and cytology results

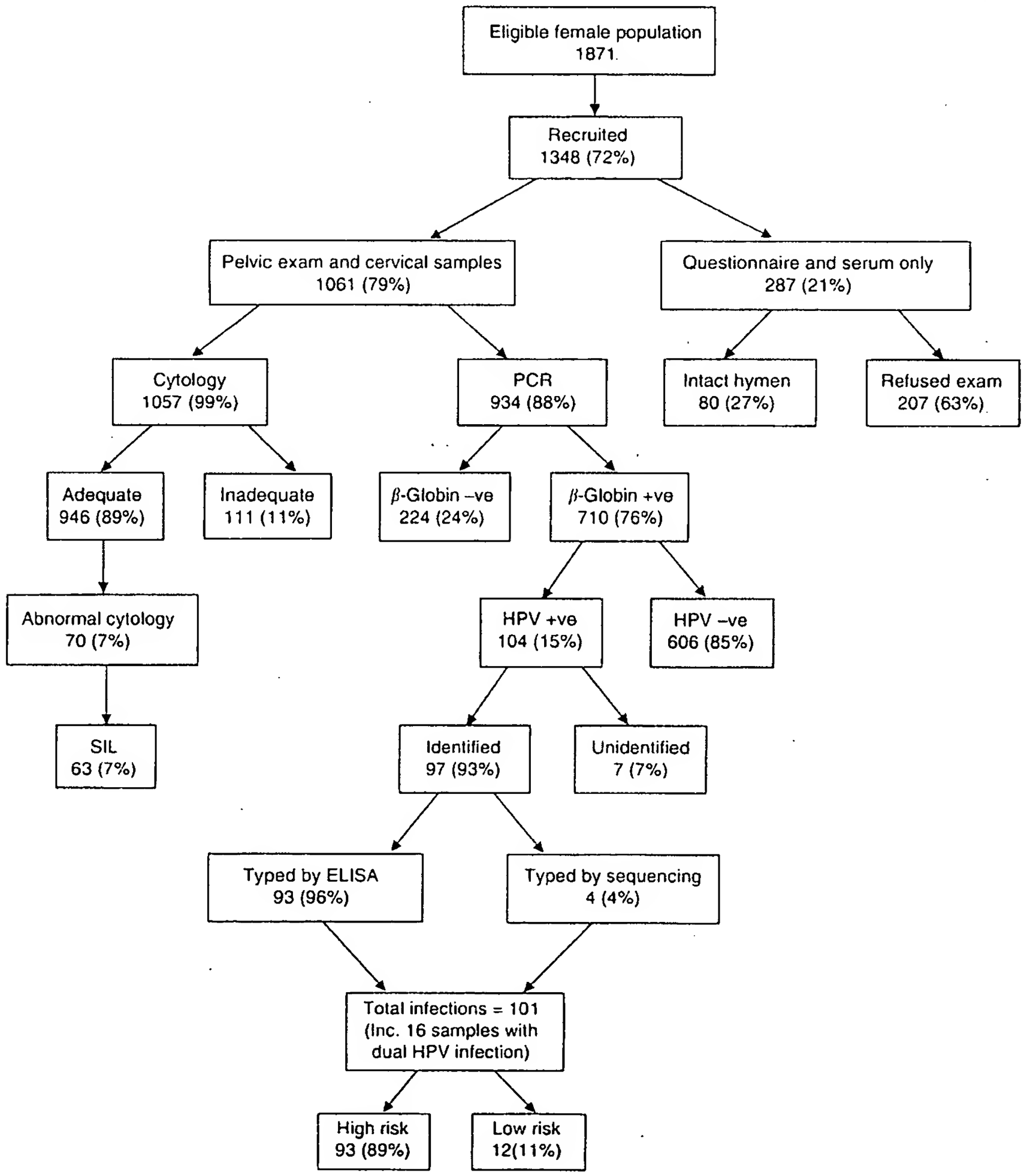

Figure 1 Overview of gambia reproductive morbidity study.

were subjected to clinical review to decide on a final diagnosis viz low- or high-grade squamous intraepithelial lesions (LSIL or HSIL) (NCI_Workshop, 1989), microinvasive or invasive carcinoma of the cervix.

Human papillomavirus detection and typing

DNA was extracted from cervical brush samples by freeze–thaw from −70°C, boiling for 10 min followed by rapid cooling on ice. Human papillomavirus DNA was detected by PCR using the consensus HPV primers GP5+ and GP6+ (biotinylated) (Jacobs *et al*, 1997). In tandem, for each sample a PCR was also set up to amplify the human β-globin gene in order to verify the presence of an adequate DNA sample and the absence of PCR inhibitors. The presence of HPV and β-globin DNA was visualised on an agarose gel. Human papillomavirus typing was preformed by enzyme linked immuno-sorbent assay (ELISA) using streptavidin coated microtitre plates to capture PCR product (Jacobs *et al*, 1997). Initially, this involved two cocktails of digoxigenin labelled probes, the first including those types commonly associated with cervical cancer (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66 and -68). The second cocktail included those types associated with benign genital lesions (HPV-6, -11, -40, -42, -43 and -44). Detection was performed using an alkaline phosphatase conjugated antidigoxigenin fab fragment. If positive for either of the two probes, a further ELISA was performed using the full spectrum of relevant (HR) or low risk (LR) individual probes.

Sequencing

Samples that could not be identified by ELISA were characterised by DNA sequencing. Selected HPV positive samples were also sequenced to confirm typing accuracy and to detect any relevant sequence variations in known HPV types. Sequencing was carried out on a Beckman Coulter CEQ2000 Automated Sequencer, using protocols and reagents supplied by the manufacturer. Human papillomavirus DNA sequencing utilised the MY09/MY11 consensus primer pair as the product of these (≈455 bp) is a more suitable target for sequencing than that of GP5+/6+ (≈140 bp). Furthermore, MY09/11 primers amplify a region containing highly conserved amino acids, which simplifies sequence alignment in HPV classification (Chan *et al*, 1995). An NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) search was carried out for each of the consensus strands produced by sequencing at least twice in each direction. Alignment analysis was carried out using the Lasergene suit of software (DNA* Inc., Madison, WI, USA). The sequences most homologous to the Gambia samples are listed in Table 2 with the percentage DNA similarity and number of differences in both amino acids (coding) and DNA only (noncoding) in the MY9/11 amplified region between the Gambia samples and the closest BLAST hits.

Statistical analysis

All data were double-entered, checked and cleaned using Epi-Info 6.4 with subsequent analysis performed in STATA 7 (Statacorp. Texas). Frequency distributions of HPV infection and different HPV types were calculated. Socio-demographic characteristics and other morbidities were examined as risk factors for HPV infection by cross tabulation and χ^2 tests. To adjust for any confounding effects, each potential risk factor was included in a logistic regression model with age group, marital status and ethnic group. The same procedure was followed for SIL. There were significant differences in the distribution of ethnic groups between the sample and the eligible population and differing disease prevalence between ethnic groups (Table 1). An adjusted prevalence was therefore calculated using the prevalence within each ethnic group and the distribution of ethnic groups in the eligible population.

RESULTS

Of 1871 eligible women, 1348 (72%) participated in the study (Figure 1) and 1061 women consented to full gynaecological examination. Of participants aged 15–24 years, 30.7% (147 out of 478) were unmarried, 80 of whom consented to intimate examination and had an intact hymen (Figure 1). These women were not examined internally by speculum and no cervical specimen was collected. No HPV sample was taken from a further 64 women examined by speculum and 63 samples were lost in transit. The β-globin human housekeeping gene was amplified in 710 out of 934 (76%) samples; consequently, only these samples were suitable for HPV analysis. The β-globin negative samples may have been incorrectly stored or damaged during transfer to the UK, prevalence figures are therefore based upon the 710 β-globin positive samples. Of the three main ethnic groups, Wolof women were slightly under-represented (38.7% of eligible population *vs* 32.1% of those with adequate cytology and 34.1% of those with adequate HPV samples) and Mandinka women were slightly over-represented (43.9% of eligible population *vs* 49.5% of those with adequate cytology and 46.2% of those with adequate HPV samples). Younger women aged 15–24 years were under-represented (27.9 and 25.9% in those with adequate cytology and adequate HPV samples, respectively, compared with 39.1% in the eligible population).

Human papillomavirus prevalence

Human papillomavirus infection was present in 95 of 710 adequate samples (crude prevalence 13.4% (95% CI: 10.96–16.11%)). Adjusting HPV prevalence for under-representation of Wolof subjects reduced prevalence slightly to 13.0%. Human papillomavirus typing by PCR-ELISA was successful in 84 samples revealing 6 different LR and 13 different HR types (Figure 2). Dual cervical HPV infection was found in 16 subjects. HPV-16 (21 out of 109 (19%)) and HPV-35 (11 out of 109 (10%) were most common. Other prevalent types were HPV-18, -33 and -58 (each 9/109 (8%)), HPV-31 (8/109 (7%)) and HPV-42 (6/109 (6%)).

DNA sequencing

Human papillomavirus DNA was amplified with the GP5+/6+ primers in 11 samples but were negative by ELISA, and therefore contained HPV types not included in the probe cocktails. These samples were sequenced with the MY 09/11 primers; five had either degenerated in storage or would not amplify, two contained multiple templates and were impossible to sequence, while four were successfully sequenced and identified but none were infected with novel types, merely types not included in the ELISA probe cocktail (Table 2).

DNA sequencing was conducted on samples successfully identified by ELISA as HPV types associated with HSIL. All HPV-16 samples sequenced showed DNA homology in the amplified region with a variant of HPV-16 African type 1 (Af1, Accession No. AF536180) (Table 2). Both HPV-18 samples sequenced displayed protein sequence homology to HPV-18 variants from Benin, West Africa (Accession Nos. U45894 and U45892) (Table 2). HPV-31 from our study showed a number of polymorphisms and silent mutations compared to the reference sequence (Accession No. J04353), but insufficient to be a novel type (Bernard, 2005) (Table 2). All HPV-33 samples sequenced displayed protein sequence homology to the reference HPV-33 strain (Accession No. M12732) (Table 2). The sequence of HPV-58

Table 1 Association between cervical HPV infection and socio-demographic, behavioural and infectious factors in the Gambia Reproductive Morbidity Study

	HPV infected/total (%)	Crude OR (95%CI)	χ^2 *P*-value	Adjusted OR[a] (95% CI)	*P*-value[b]
Age group					
15–24	27/184 (15%)	1		1	
25–34	22/213 (10%)	0.67 (0.37–1.22)		0.69 (0.37–1.29)	
35–44	27/191 (14%)	0.96 (0.54–1.7)		1.10 (0.60–2.04)	
45–54	19/122 (16%)	1.07 (0.57–2.02)	0.461	1.13 (0.57–2.25)	0.387
Ethnic group[c]					
Wolof	20/242 (8%)	1		1	
Mandinka	47/328 (14%)	1.86 (1.07–3.22)	0.002	1.76 (1.00–3.09)	
Fula	27/129 (21%)	2.94 (1.57–5.48)		2.85 (1.51–5.40)	0.005
Marital status[c]					
Monogamous	40/266 (15%)	1		1	
Polygamous	51/416 (12%)	0.79 (0.50–1.23)		0.84 (0.51–1.36)	
Divorced/widowed	3/22 (14%)	0.89 (0.25–3.15)	0.582	0.84 (0.23–3.07)	0.772
Parity					
Nulliparous	9/56 (16%)	1		1	
Para 1–3	29/205 (14%)	0.86 (0.38–1.94)		0.85 (0.37–1.96)	
Para 4–7	40/322 (12%)	0.74 (0.34–1.63)		0.83 (0.33–2.08)	
Para 8+	17/127 (13%)	0.81 (0.34–1.94)	0.871	0.65 (0.23–1.82)	0.844
Sexually active in last 3 months					
Yes	62/497 (12%)	1		1	
No	21/133 (16%)	1.31 (0.77–2.25)		1.23 (0.70–2.19)	
No answer	12/80 (15%)	1.23 (0.63–2.42)	0.549	1.19 (0.58–2.41)	0.731
Genital prolapse					
Absent	59/377 (16%)	1		1	
Present	36/329 (11%)	0.66 (0.42–1.03)	0.067	0.73 (0.46–1.16)	0.183
HSV2 serology					
Negative	53/444 (12%)	1		1	
Positive	40/244 (16%)	1.45 (0.93–2.26)	0.102	1.27 (0.78–2.08)	0.343
Current STI[d]					
Absent	81/605 (13%)	1		1	
Present	10/73 (14%)	1.03 (0.51–2.08)	0.941	0.77 (0.36–1.65)	0.494
Endogenous infection[e]					
Absent	48/328 (15%)	1		1	
Present	45/319 (14%)	0.96 (0.62–1.49)	0.848	1.00 (0.63–1.59)	0.985
Anaemia[f]					
Absent	41/322 (13%)	1		1	
Mild	33/272 (12%)	0.95 (0.58–1.54)		0.86 (0.52–1.43)	
Moderate/severe	18/96 (19%)	1.58 (0.86–2.91)	0.237	1.45 (0.77–2.73)	0.319

[a]Adjusted for age group, marital status and ethnic group. [b]From likelihood ratio test adjusting for age group, marital status and ethnic group. [c]Five single women (one had HPV) and 11 women of other ethnic groups (one had HPV) were excluded from this analysis because of small numbers. [d]Chlamydia trachomatis, Trichomonas vaginalis, positive Syphilis serology. [e]Candida culture positive, Bacterial vaginosis (Nugent's criteria). [f]Anaemia: mild = Hb < 11 (pregnant) Hb < 12 (nonpregnant), moderate/severe = Hb < 9 (pregnant), Hb < 10 (nonpregnant).

from our study was identical to a variant isolated in the West African nation of Mali (Table 2).

Cytology and histology

The overall crude prevalence of cervical precancer was 6.7% (63/946 adequate smears (95% CI: 5.2–8.4%)), or 6.5% after adjusting for the under-representation of Wolof women Cytological abnormalities were confirmed by histology in 55.6% (35/63) of cases with HSIL present in 2.3% ($n = 22$; 16 confirmed by histology), LSIL were found in 3.3% ($n = 31$; 19 confirmed by histology) and atypical squamous cells of uncertain significance (ASCUS) in 1.1% ($n = 10$). A single case of invasive cervical cancer was diagnosed by clinical and cytological criteria but the woman sadly died before the histology could be confirmed; it was included in the HSIL analysis.

Epidemiology

Risk factors for cervical precancer included age (χ^2 $P = 0.03$) and cervical HPV infection. Rates of SIL were highest at ages 25–34 years (22 out of 265 (8%)) and 35–44 years (24/268 (9%)), intermediate at ages 45–54 (9/150 (6%)) and lowest at ages 15–24 (8/263 (3%)). In subjects where both adequate cytology/histology and HPV analysis were available, 574/612 (93%) had normal cytology, 38 out of 612 (6%) had SIL and seven out of 612 (1%) had ASCUS. Of the subjects with normal cytology 525/574 (91%) were negative for HPV while in those with SIL/ASCUS 20/38 (53%) had

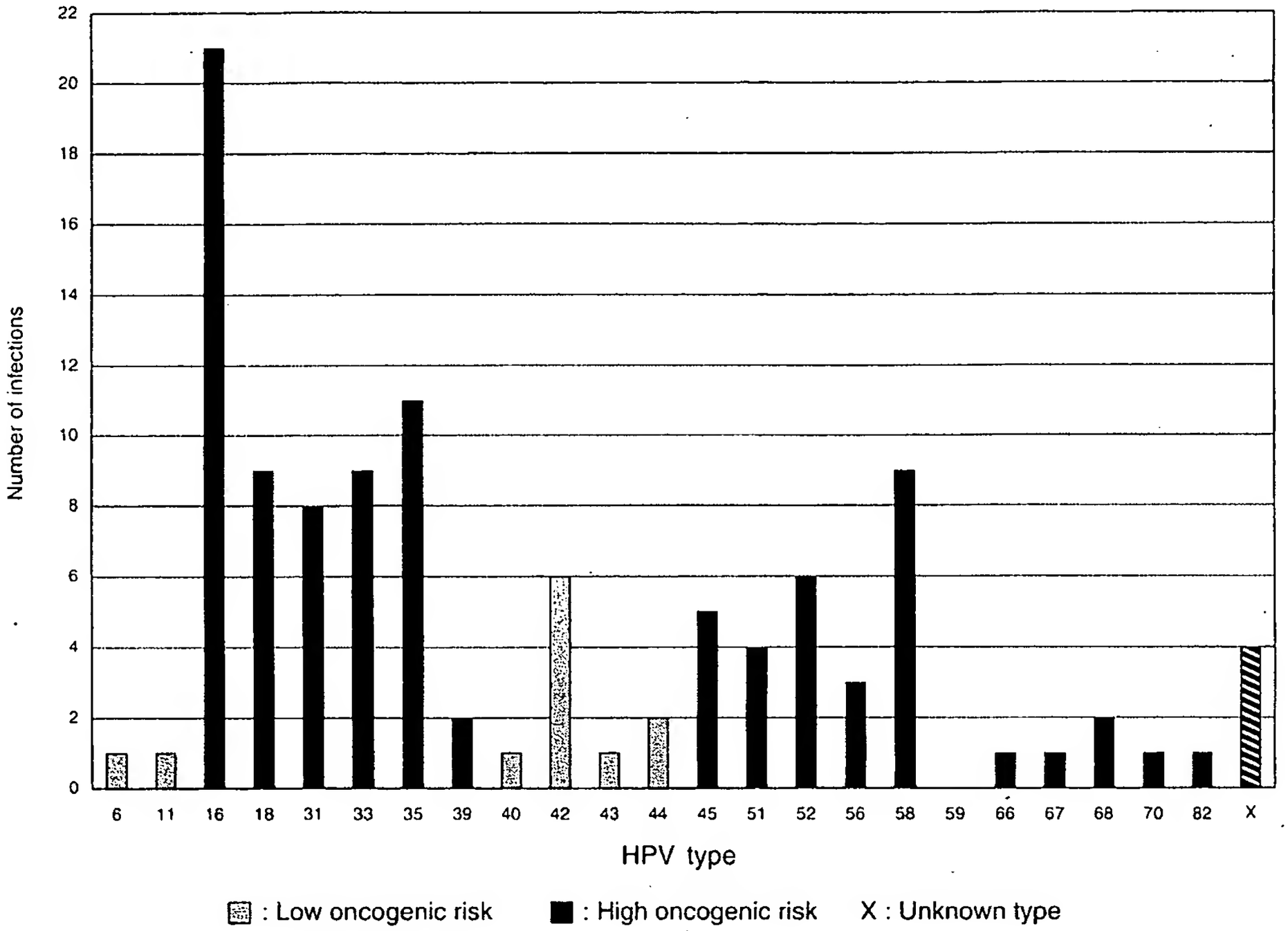

Figure 2 Total of all HPV types (inc. multiple infections).

HR HPV types. Of subjects with HSIL, 13/15 (87%) had HR HPV types, although the other two were negative for HPV. Among subjects with HSIL, HPV-16, HPV-33 and HPV-58 were most common, each found in 3/15 (20%). HPV-16 was found in the single invasive cancer case. HPV-18, -31, -35, -39 and -45 occurred once each among subjects with HSIL. Of 30 subjects with LSIL/ASCUS, seven were infected with HR HPV types; of subjects with normal cytology 66/576 (12%) had HPV infection.

No associations were found between HPV and other current sexually transmitted infections (STI) (Table 1). Moderate or severe anaemia was associated with HPV infection, but not significantly. Similarly, abnormal vaginal bleeding or discharge, pelvic masses and body mass index showed no significant associations with HPV infection. Of 1110 subjects, only 61 (6%) used modern methods of contraception, too few to examine associations with HPV. Among socio-demographic factors, only the subject's ethnic origin was significantly associated with HPV infection, with Mandinka (14%) and Fula (21%) women having a higher risk of cervical HPV infection than Wolof subjects (8%) (Table 1).

DISCUSSION

This first unselected study of cervical HPV and SIL in an unscreened, rural West-African population of its kind shows HPV infection to be common at 13.4% (adjusted = 13.0%) with the majority HR types. Although younger subjects were under-represented, such an age profile is comparable with other studies. The high cervical HPV infection prevalence and SIL are in agreement with Gambian Cancer Registry data (Koulibaly *et al*, 1997; Bah *et al*, 2001). Comparable prevalences of 14% have been observed in two cytologically normal populations in Senegal, the only nation on which The Gambia borders (Astori *et al*, 1999; Xi *et al*, 2003). A recent study from Nigeria, West Africa, of an unselected population of similar size typed by GP5 + / 6 + PCR-ELISA, found an HPV prevalence of 26% (Thomas *et al*, 2004). However, the population in Thomas *et al* was urban (Ibadan, pop. >1M) and the higher HPV prevalence may be explained by differing sexual behaviour as well as the much higher prevalence of LR HPV types in the Nigerian study than in our study.

Much higher HPV prevalence figures have been reported in recent unselected studies from Eastern and Southern Africa, ranging from 34% in rural Zimbabwe (Baay *et al*, 2004) to 44% in urban Kenya (De Vuyst *et al*, 2003). Human immunodeficiency virus (HIV) infection and concomitant immune suppression is an acknowledged cofactor in the progression of cervical cancer (Feingold *et al*, 1990; Moscicki *et al*, 2004a, b) and such high HPV prevalence may be due to the high rates of HIV infection in these regions (UNAIDS, 2004). The Gambia has one of the lowest HIV infection rates in Africa (Ramsay, 1993, [73]Da Costa, 1994; Schim van der Loeff *et al*, 2003). Multiple cervical HPV infection is common in Africa (Chabaud *et al*, 1996; Castellsague *et al*, 2001; Gravitt *et al*, 2002; Stanczuk *et al*, 2003; Xi *et al*, 2003; Baay *et al*, 2004) yet we encountered no more than two co-infections, perhaps because of the low HIV rates in The Gambia (UNAIDS, 2004). Multiple HPV infection have been linked to HIV immunosuppression (Levi *et al*, 2002a, b; Moscicki *et al*, 2004a)

Table 2 Genetic comparison of HPV DNA sequences in the Gambia Reproductive Morbidity Study compared to closest Genbank entries

RMS Gambia sample	Genbank accession number	Isolate number (type)	Origin	Homology to Gambian sample(s)		
				% DNA similarity	Noncoding variations	Coding variations
Samples identified by sequencing						
2006D	U01532	AE2 (MY9/11)	USA	100	0	0
	U12481	IS039 (MY9/11)	Argentina	99.8	1	0
	AF293961	AE2/IS039 (Full)	New York, USA	99.5	2	0
	AB027021	HPV-82 (Ref.)	Japan	89.0	38	8
2712F	D21208	HPV-67 (Ref.)	NK	98.3	4	3
	U12492	(MY09/11)	NK	97.8	6	3
2781F	U01535	AE1 (MY09/11)	USA	99.5	2	0
	U21941	HPV-70 (Ref.)	Sweden	99.3	3	0
	U12476	CP141 (MY09/11)	New Mexico, USA	99.1	3	1
	U12486	LVX160 (MY09/11)	Indigenous Amazonian	99.1	3	1
2919F	AF538717	SDL105 (MY09/11)	Minesota, USA	99.0	4	0
	Y17206	GA115 (MY09/11)	Senegal[c]	99.8	1	0
	M73258	ME180 (Cell Line)	NK	98.3	5	2
	U45934	IS362 (MY09/11)	Germany	97.8	7	2
	X67161	HPV-68 (Ref.)	NK	93.0	22	7
Samples identified by PCR-ELISA						
HPV-16	AF536180	Af1 variant (Full)	Africa	100	0	0
	U34188	OR7587 (L1)	USA	100	0	0
	AF472508	Af1 Variant (Full)	Africa	99.8	1	0
	U34189	OR7632 (L1)	USA	99.8	1	0
	U34183	OR6106 (L1)	USA	99.5	1	1
	AF472509	Af2 (Full)	Africa	99.3	2	1
	AF134178	GU2 (L1)	NK	99.3	2	1
	U37217	(L1+L2)	Zaire[d]	99.3	2	1
	U34186	OR7145 (L1)	USA	99.3	2	1
		HPV16R (Ref.)	Composite	99.0	4	0
HPV-18	U45894	IS172 (MY09/11)	Benin[c]	100	0	0
	U45892	IS168 (MY09/11)	Benin[c]	99.8	1	0
	U45893	IS768 (MY09/11)	Uganda[c]	99.3	2	1
		0069A[a] (MY09/11)	Gambia	99.5	2	0
	X05015	HPV-18R (Ref.)	Brazil	97.6	8	2
HPV-31	U37410	(L1 & L2)	NK	99.0	3	1
	J04353	HPV-31 (Ref.)	NK	98.6	5	1
HPV-33	U45897	IS827 (MY09/11)	Guinea[c]	99.8	1	0
		0184C[b] (MY09/11)	Gambia	99.8	0	1
	M12732	HPV-33 (Ref.)	NK	99.8	1	0
HPV-58	U45928	IS417 (MY09/11)	Mali	100	0	0
	AY101598	Bsb-2 (MY09/11)	Brazil	99.7	0	1
	U45929	IS404 (MY09/11)	Mali	99.2	1	2
	D90400	HPV-58 (Ref.)	Japan	98.9	1	3

[a]Sample from this study differing in two noncoding bases from the other HPV-18 samples sequenced. [b]Sample from this study differing in one coding base from the other HPV-33 samples sequenced. [c]West Africa. [d]Central Africa.

and in Zimbabwean HIV infected individuals may exhibit quintuple HPV infection (Baay *et al*, 2004).

High oncogenic risk HPV can be detected in >99.7% of high-grade pre-invasive and invasive cervical lesions (Walboomers *et al*, 1999). In this study, HPV was found in 87% of subjects with high-grade lesions, indicating a high degree of accuracy for HPV detection. Failure to detect HPV in two subjects with HSIL may be due to integration of viral DNA into their genomic DNA and the consequent deletion of the target site for the consensus PCR primers. HPV shows a wide type distribution (Figure 2) (eight types associated with HSIL), but an even distribution among subjects. HPV-16, the most common HR type worldwide (Bosch, 1995a, b; Muñoz *et al*, 2003) is the most common type in this study, showing the strongest association with precancer.

The HR type HPV-35 was the second most prevalent type (10%) in this study and meta-analysis of worldwide HPV prevalence studies shows low overall prevalence of HPV-35 (≈2%), even in Africa (Clifford *et al*, 2003b). Three selective urban Senegalese studies found a very low prevalence of HPV-35 ranging from no HPV-35 positive samples (Astori *et al*, 1999; Chabaud *et al*, 1996) to 1% in the most recent (Xi *et al*, 2003). Inconsistency between studies examining Senegal and Gambian populations with overlapping ethnic groups, religious practices and trade routes initially appears incongruous. However, the GP5+/6+ primers used in the Gambian study are estimated to be 5000 times more sensitive in the detection of HPV-35 (Qu *et al*, 1997) than the MY09/MY11 primer pair used in the Xi and Astori studies. Therefore, the true HPV-35 prevalence in these Senegalese studies is likely to have been underestimated. This is supported by other African studies where HPV-35 is among the four of the most prevalent types in studies not exclusively using the MY09/11 primer pair for HPV typing (Castellsague *et al*, 2001; De Vuyst *et al*, 2003; Baay *et al*, 2004; Naucler *et al*, 2004; Thomas *et al*, 2004). The Nigerian population displays a similar distribution of HR types to the

Gambian except that HPV-16 and -35 are jointly the most prevalent types in Nigerian study (Thomas *et al*, 2004). It thus appears that HPV-35 is prevalent throughout sub-Saharan Africa but the extent of this prevalence is underestimated by the use of the MY09/11 primer pair. This indicates the importance of methodology in the design of viral epidemiological studies. Of the 13% (12/93) of samples positive for LR HPV, HPV-42 was most prevalent, accounting for 50% of LR types and 6% of all HPV types. This is in contrast to the USA and Europe where HPV-6 and HPV-11 are the dominant LR types (Muñoz *et al*, 2003) and most other African studies, where HPV-53 and -54 are the dominant LR types (Castellsague *et al*, 2001; De Vuyst *et al*, 2003; Xi *et al*, 2003). HPV-42 was the most prevalent LR type and overall the most common HPV type in Nigeria (Thomas *et al*, 2004), increasing the overall HPV prevalence, with a LR:HR HPV ratio of 35:65 compared to 11:89 in our Gambian study.

Human papillomavirus is classified into types, subtypes and variants by comparative DNA homology based upon the L1 outer capsid. To define a new HPV type it must have less than 90% L1 DNA homology to any previously defined types. Human papillomavirus types can be further characterised into subtypes, 90–98% L1 DNA homology, and variants (either >98% L1 DNA homology or oncogene variations). No HPV samples in this study were found to be novel types after identification by sequencing, although some were unusual subtypes or variants (Table 2). The DNA sequences of HPV-16, -18, -33 and -68 revealed HPV variants found by other African studies. In particular, the Gambian HPV-68 samples are an exact DNA match to an HPV-68 subtype first isolated from a study in neighbouring Senegal (Astori *et al*, 1999). Our study also confirms the importance of HPV-16 Af1, the most prevalent of the HPV-16 subtypes in Africa (Yamada *et al*, 1997). Gambian HPV-18 and HPV-33 variants are homologous to those found in Benin, Uganda and Guinea (Table 2). Thus, with the non-HPV-16 samples associated with HSIL in this study, a number of polymorphisms are observed compared to the reference types but most are identical or similar to types found in other African studies (Table 2).

Histologically confirmed cervical neoplasia prevalence in our study was 6.7% (adjusted = 6.5%). Diagnostic accuracy was improved by classifying subjects with abnormal cytology but normal histology as 'normal cervix'. The prevalence of abnormal cytology alone was slightly higher at 7.4% (70/946 adequate smears). Other investigators have used a cytological screening to identify cervical disease providing a less accurate prevalence of SIL (Herrero *et al*, 2000; Castellsague *et al*, 2001).

In this study, HPV infection was not associated with age, parity or concurrent STI. The even distribution of HPV prevalence at different ages is unusual though observed in other developing countries such as India (Franceschi, 2005) and Argentina (Matos *et al*, 2003). Studies from industrialised countries show a high HPV prevalence among women under 25 years declining with age (Sellors *et al*, 2000; Giuliano *et al*, 2001). This may be explained by study bias since data are commonly collected from such highly selective populations as college students or clinic attendees. Our study, however, concerns a nonselective population with minimal migration; differing sexual behaviour in different cultural environments may contribute to the variable age-profiles. Certain ethnicities were associated with higher risk of cervical HPV infection in Gambia, a decreased risk being seen in Wolof women compared with Mandinka and Fula subjects, perhaps due to differing patterns of sexual behaviour. Another possibility is genetic variation in susceptibility to HPV infection, for example, due to differences in HLA types while differences in female genital cutting (FGC) between the ethnic groups suggest a further mechanism. The Wolof are the only ethnic group in our study that do not practice FGC and may thereby be less susceptible to HPV infection. A previous study of this group found lower prevalences of HSV-2 (herpes simplex virus) and bacterial vaginosis among nongenitally cut women (Morison *et al*, 2001).

The ideal treatment strategy for this rural Gambian population would involve the use of a prophylactic vaccine to prevent cellular viral entry. Current prophylactic vaccine formulations require three needle injections, cold storage and offer only type specific protection (Koutsky *et al*, 2002; Harper *et al*, 2004). In rural Gambia with a spread of prevalent HR HPV types, where funding is short supply, this is impractical. However, vaccines currently in development include oral based prophylactic vaccines (Baud *et al*, 2004; Berg *et al*, 2005; Sasagawa *et al*, 2005), which will be easier to store and administer and thus economically viable.

The ethnic groups in our study population have not previously been studied using these methods. Other studies in African populations using the same methods to those used in our study (La Ruche *et al*, 1998; Bayo *et al*, 2002; Baay *et al*, 2004; Naucler *et al*, 2004; Thomas *et al*, 2004) show differing LR/HR ratios and disease associations. This suggests that in addition to HPV infection host factors are central in the development of cervical cancer. Our findings strengthen existing data from developing nations, showing widespread cervical HPV infection of a broader spectrum than found in industrialised nations with HPV-16 most common. An effective vaccine for the Gambia must be multivalent and include the other HR types prevalent in this representative rural population including HPV-18, -58 and -33. HPV-35 was not found in high-grade lesions and evidence for its oncogenicity is limited. However, as the second most prevalent type in this study with close homology to HPV-16, it may be worth including in any potential vaccine.

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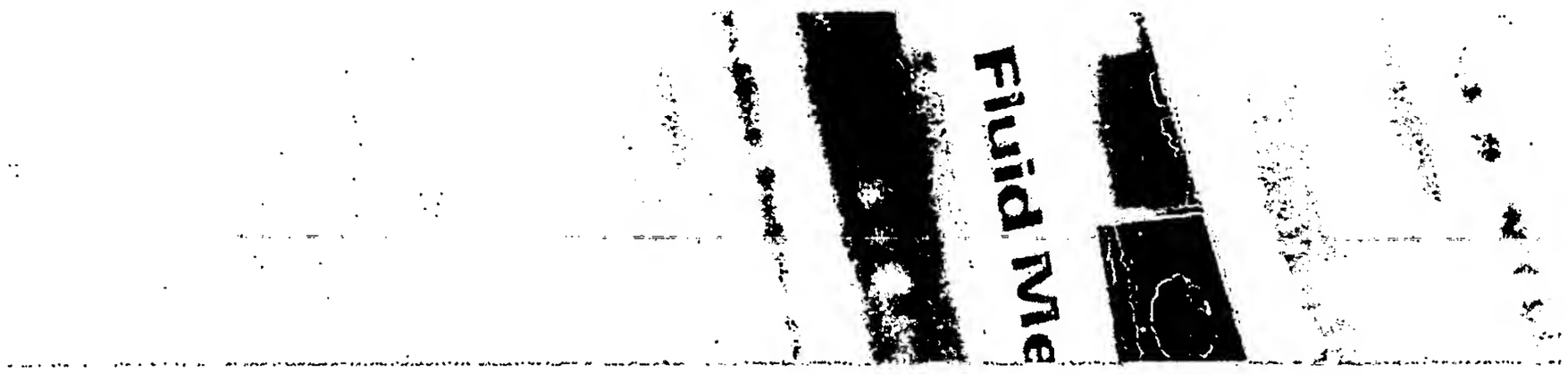

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Comparison of DNA sequencing and roche linear array® in human papillomavirus (HPV) genotyping

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Résumé / Abstract
Background: Human papillomavirus (HPV) is the etiological agent of cervical cancer. HPV genotyping is important to determine the presence of high-risk types. Recently, a new HPV genotyping method, the Roche Linear array® genotyping test, was introduced and is compared here with a sequencing-based HPV genotyping system. Materials and Methods: A series of 102 women (age range 30-55 years) shown to be HPV DNA-positive by PCR were typed by sequencing and the Linear array genotyping assay. Results: The sequence analysis revealed the presence of 80 single high-risk types and 22 single low-risk types. With the Linear array, single infections were found in 46 cases, double infections in 37 cases, triple infections in 12 cases, and more than three in 6 cases. One case positive by sequencing gave a negative result by Linear array. Altogether, a concordant single genotype was found in 93 (91.2%) out of the 102 cases and the single-type concordance between the two assays was significant (Spearman rho =0.849, p=0.0001; intraclass correlation coefficient (ICC) (ICC=0.924, 95%CI 0.888-0.949) (p=0.0001). The majority of the disparate results were due to the detection of multiple types by the Linear array. Conclusion: The Roche Linear array® is a highly accurate assay for HPV genotyping. This is particularly true in the presence of multiple infections which DNA sequencing is unable to resolve.

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Routine human papillomavirus genotyping by DNA sequencing in community hospital laboratories

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Abstract

Background: Human papillomavirus (HPV) genotyping is important for following up patients with persistent HPV infection and for evaluation of prevention strategy for the individual patients to be immunized with type-specific HPV vaccines. The aim of this study was to optimize a robust "low-temperature" (LoTemp™) PCR system to streamline the research protocols for HPV DNA nested PCR-amplification followed by genotyping with direct DNA sequencing. The protocol optimization facilitates transferring this molecular technology into clinical laboratory practice. In particular, lowering the temperature by 10°C at each step of thermocycling during *in vitro* DNA amplification yields more homogeneous PCR products. With this protocol, template purification before enzymatic cycle primer extensions is no longer necessary.

Results: The HPV genomic DNA extracted from liquid-based alcohol-preserved cervicovaginal cells was first amplified by the consensus MY09/MY11 primer pair followed by nested PCR with GP5+/GP6+ primers. The 150 bp nested PCR products were subjected to direct DNA sequencing. The hypervariable 34–50 bp DNA sequence downstream of the GP5+ primer site was compared to the known HPV DNA sequences stored in the GenBank using on-line BLAST for genotyping. The LoTemp™ ready-to-use PCR polymerase reagents proved to be stable at room temperature for at least 6 weeks. Nested PCR detected 107 isolates of HPV in 513 cervicovaginal clinical samples, all validated by DNA sequencing. HPV-16 was the most prevalent genotype constituting 29 of 107 positive cases (27.2%), followed by HPV-56 (8.5%). For comparison, Digene HC2 test detected 62.6% of the 107 HPV isolates and returned 11 (37.9%) of the 29 HPV-16 positive cases as "positive for high-risk HPV".

Conclusion: The LoTemp™ ready-to-use PCR polymerase system which allows thermocycling at 85°C for denaturing, 40°C for annealing and 65°C for primer extension can be adapted for target HPV DNA amplification by nested PCR and for preparation of clinical materials for genotyping by direct DNA sequencing. HPV genotyping is performed by on-line BLAST algorithm of a hypervariable L1 region. The DNA sequence is included in each report to the physician for comparison in following up patients with persistent HPV infection, a recognized tumor promoter in cancer induction.

Background

Human papillomavirus (HPV) testing was introduced to compensate for the poor sensitivity and specificity of the Pap smear cytology often used as diagnostic tool for borderline precancerous lesions [1]. Digene Hybrid Capture 2 (HC2) test, the only test approved by the U.S. Food and Drug Administration (FDA), is commonly used to determine if a cervicovaginal cell suspension contains "high-risk" oncogenic HPVs [2], often functioning as a triage for colposcopic evaluation of the cytologically borderline cases [3-5]. However, it is now recognized that persistent infection of a "high-risk" HPV, not the mere presence of the HPV virus itself, is the pivotal promoter in causing cervical precancerous lesions and cancer [6-9]. Most of HPV infections, even caused by "high-risk" genotypes, are transient with normal Pap cytology in sexually active young women [10-13]. In 93% of initially infected women, the same viral type is not detected upon re-examination four menstrual cycles later [14]. The median duration of positivity detectable by PCR for a specific HPV type in these young women is 168 days [15]. Multiple "high-risk" HPV infections do not constitute a higher risk for the development of cervical neoplasia when compared with single persistent high-risk HPV infection [16]. For the development and maintenance of a high-grade squamous intraepithelial lesion (SIL), the risk is greatest in women positive for the same genotype of HPV on repeated testing [6-8]. Viral load is not a useful parameter to predict high-grade SIL [17]. High-grade SIL is often associated with a viral DNA load lower than that observed in less severely affected cells [18].

In view of the recent advance in the understanding of the relationship between persistent HPV infection and cervical neoplasia, a sensitive and specific technology to detect and accurately genotype HPV is needed for clinical management of persistent infections. The HC2 test cannot be converted to a genotyping assay and is associated with a significant number of false-negative and false-positive results when compared with other more stringent PCR-based HPV genotyping assays [19-23]. It is reported to generate 25% false-negative results in cases with biopsy-proven high-grade SIL even when all these biopsies have been proven to contain high-risk HPV DNA by PCR [24]. "The lack of multiple, competitive, well-validated tests" for HPV assay is quoted as being "a problem" in formulating new guidelines in management of cervical abnormalities [25].

The introduction of the type-specific Gardasil™ HPV vaccines into the sexually active female population also requires genotype monitoring of the HPV infections before and after immunization to develop prevention strategy for the individual patients. Based on a "Background Document" submitted to the FDA [26], injection of HPV vaccines into women who have concurrent vaccine-relevant HPV type infections may increase the risk, by 44.6%, of developing high-grade precancerous lesions in the cervix. Therefore, it would be prudent to perform a genotype-specific HPV assay if prior HPV infection is suspected.

Target nested PCR amplification of a conserved region of the HPV L1 gene DNA with the consensus MY09/MY11 and GP5+/GP6+ primers, or their equivalent, followed by genotyping with direct DNA sequencing is a generally accepted scientific tool in research [21,22,27]. However, it has not been used in clinical laboratories because handling the temperature-sensitive PCR reagents and the requirement for template purification in the PCR protocols are too labor-intensive for routine applications. This paper records our experience in using a high-processivity, room temperature-stable robust DNA polymerase system to facilitate the transfer of this molecular technology into clinical laboratory practice. Each HPV-positive result is validated by genotyping with direct DNA sequencing of a hypervariable region of the L1 gene. The HPV DNA sequencing information can be included in the laboratory report for future clinical follow-up of persistent infections.

Methods

Since the PCR conditions adapted for this protocol depended on the use of a new low-temperature ready-to-use moderately thermostable DNA polymerase system, the sensitivity and specificity of the LoTemp™ HiFi® DNA polymerase in amplification of HPV DNA were first validated and compared with those obtained by standard heat-resistant *Taq* polymerases available on the market.

The purified full-length plasmid DNAs of HPV types -16, -18 and -6B purchased from American Type Culture Collection (ATCC) were used as standards for method development. Then the HPV type-16 DNA was used as a routine positive control. Molecular grade pure water instead of DNA extract was used as negative control.

Theoretical sensitivity of the PCR system chosen for this study was determined by using serial 10-fold dilutions of the ATCC-certified HPV standard containing 200 ng of plasmid DNA of HPV-16, -18 and -6B with TE buffer to single copy of genomic DNA per μL as the template to run primary and nested PCR on each dilution in duplicate. The theoretical number of copies of HPV DNA in the template used for each MY09/MY11 PCR was calculated according to a generally accepted conversion formula [28]. All nested PCR products were confirmed by DNA sequencing to be those of respective HPV genotypes expected.

For *Taq* PCR amplifications, the reaction mixture of 25 μL contained 100 mM KCl, 20 mM Tris-HCl pH 8.0, 25 mM $MgCl_2$, 2.5 mM of each dNTP, 2.5 units of Takara Taq polymerase (Takara Bio Inc., Shiga, Japan), 100 pmol of each consensus primer (MY09/MY11 or GP5+/GP6+) and 1 μL of HPV DNA at various dilutions in TE buffer (or if for nested PCR, 1 μL of the MY09/MY11 PCR products). The reaction mixture was subjected to 35 cycles of amplification in an MJ Research thermocycler (Waltham, MA). Each cycle consisted of a denaturing step at 94°C for 0.5 min, an annealing step at 55°C for 1 min, and a chain elongation step at 72°C for 1 min.

In LoTemp™ HiFi® DNA polymerase PCR, the protocol described below for clinical specimens was followed except that a 35-cycle amplification was used for comparison with *Taq* PCR amplification.

The clinical samples used for this study were 515 alcohol-preserved liquid-based cervicovaginal cytology specimens (Cytyc or Surepath) submitted by physicians in the New Haven, Connecticut area as part of routine gynecologic examinations. Age distribution of the patients and the cervical pathologic conditions were not the subjects of this study. Digene HC2 test for high-risk HPV ordered by the physicians was performed routinely on each sample by one of the two independent clinical laboratories (Quest Diagnostics Laboratory, Wallingford or Pathology & Laboratory Services, LLC, Woodbridge, CT) according to instructions provided by Digene Corporation (Gaithersburg MD). In general the patients were women below age 30 who had a Pap cytology diagnosis of atypical squamous cells of undermined significance (ASCUS) or women 30 years and older regardless of the Pap cytology findings [4].

After the material was taken from each sample for routine cytology and HC2 test, about 1 mL of the cell suspensions was placed in a 1.5 mL Eppendorf tube, blind-coded with a case number and transferred to the laboratory at Milford Hospital for HPV PCR/DNA sequencing.

DNA extraction from the alcohol-fixed cells was accomplished according to a National Cancer Institute (NCI) protocol [29] with minor modification. Briefly, the cell suspension was first centrifuged in an Eppendorf microcentrifuge (model 5424) equipped with a rotor (model FA45-24-11) for 5 min at 13,000 rpm. The cells in the pellet were washed in 1 mL reagent grade water and then in 1 mL buffer consisting of 50 mM Tris-HCl, 1 mM EDTA, 0.5% Tween 20, pH 8.1. The washed cell pellet was re-suspended and digested at 45–55°C overnight in 100 μL of 0.1 mg/mL proteinase K (Sigma Chemical Co., St. Louis, MO) dissolved in the same washing buffer. After denaturing the proteins in the cell digestate in a metal block heated to 95°C for 10 min and a final centrifugation of the digestate at 13,000 rpm for 5 min, the supernatant was carefully pipetted out and placed in a clean microcentrifuge tube to be used for PCR without further purification or stored at -20°C.

The general methodology of primary PCR amplification of a 450 bp segment of the HPV L1 gene with a pair of consensus MY09/MY11 primers followed by nested PCR with a pair of GP5+/GP6+ general primers was used for HPV DNA preparation. The 150 bp nested PCR products in the positive specimens were genotyped by direct DNA sequencing [21,22] with minor modifications briefly summarized as follows.

For primary PCR amplification, 1 μL of the DNA extract, 1 μL of 10 μmolar MY09 primer, 1 μL of 10 μmolar MY11 primer and 2 μL of water were added to a PCR tube containing 20 μL of LoTemp™ HiFi® DNA polymerase ready-to-use mix (HiFi DNA Tech, LLC, Trumbull, CT) which contains all the components needed for low temperature PCR, including dNTPs, Mg^{++}, buffer, HiFi® DNA polymerases, proprietary dsDNA melting agents and dNTP preservatives, to reach a final 25 μL reaction volume. For thermocycling, the temperature steps of a TC-412 Thermal Cycler (Techne Incorporated, Burlington, NJ) were programmed for an initial heating at 85°C for 2 min, followed by 30 cycles at 85°C for 30 sec, 40°C for 30 sec, and 65°C for 1 min. The final extension was 65°C for 10 min.

For nested PCR, a "trace" of the MY09/MY11 PCR products was transferred by a glass rod with clean wettable surface of about 1.5 mm in diameter to a second PCR tube containing 25 μL of complete nested PCR reaction mixture consisting of 20 μL of LoTemp™ HiFi® DNA polymerase ready-to-use mix, 1 μL of 10 μmolar GP5+ primer, 1 μL of 10 μmolar GP6+ primer and 3 μL of water, using the same thermocycling program as described above.

After completion of the primary and the nested PCR, a 5 μL aliquot of the PCR products was pipetted out from each tube and mixed with 2 μL loading fluid for electrophoresis in a 2% agarose gel containing ethidium bromide. The gel was examined under UV light. Visualization of a 450 bp PCR product band in the MY09/MY11 lane and/or a 150 bp band in the nested PCR lane on the agarose gel provided evidence of HPV DNA in the sample, pending genotyping with direct DNA sequencing as a means of final validation.

For DNA sequencing, 1 μL of the nested PCR products, if positive, was pipetted out from the nested PCR tube for direct DNA sequencing, using 1 μL of 5 μmolar GP6+ primer as the sequencing primer, 1 μL of the BigDye® Ter-

minator (v 1.1/Sequencing Standard Kit), 3.5 μL 5× buffer, and 13.5 μL water in a total volume of 20 μL for 20 enzymatic primer extension/termination reaction cycles in an ABI thermocylcer Model 9600 according to the protocol supplied by the manufacturer (Applied Biosystems). After dye-terminator cleanup with a Centri-Sep column (Princeton Separations, Adelphia, NJ), the reaction mixture was loaded in an automated ABI 3130 four-capillary Genetic Analyzer for sequence analysis. Sequence alignments were performed against various standard HPV genotype sequences stored in the GenBank database by online BLAST analysis to arrive at specific genotyping.

One μL of each DNA extract was placed in a separate PCR tube with a β-globin primer pair [30] for human genomic DNA amplification as a control of specimen adequacy. The primers for β-globin gene amplification were 1 μL of 80 μmolar 5'-ACACAACTGTGTTCACTAGC and 1 μL of 80 μmolar 5'-CAACTTCATCCACGTTCACC in a 20 μL of LoTemp™ HiFi® DNA polymerase ready-to-use mix with 2 μL water added. The LoTemp™ thermoclycling program mentioned above was used.

Three (3) PCR tubes per sample were used routinely for the β-globin gene, the MY09/MY11 primer and the GP5+/GP6+ nested amplification, respectively.

To test performance reproducibility of this PCR/DNA sequencing procedure, aliquots of the digestate of 30 individual clinical samples were tested in parallel duplicate runs. The duplicate results of the three PCRs on each case and the genotyping results of the positive cases by DNA sequencing were compared.

Samples that did not show an MY09/MY11 or a GP5+/GP6+ PCR band, but showed evidence of positive β-globin gene amplification were interpreted as HPV-negative. Specimens that did not show any PCR products in all three lanes were considered unsatisfactory for evaluation due to low DNA extraction or presence of a PCR inhibitor. There were two (2) unsatisfactory cases among a total of 515 processed. The remaining 513 cases were accepted as satisfactory for analysis. Of these 513 cases, 107 were positive for nested PCR products, all proven to be those of HPV DNA by direct DNA sequencing, using GP6+ as the sequencing primer.

The samples infected with more than one genotype of HPV were indicated by the appearance of numerous ambiguous or overlapping peaks in the DNA sequencing tracings. For each of these mixed infections, the nested PCR products were subjected to additional four individual primer extension/termination reactions to rule out infection by the Gardasil™ vaccine-relevant HPV, namely HPV types -16, -18, -6 or -11, using the following type-specific primers [31].

HPV-16 type-specific sequencing primer 5'-GCTGCCATATCTACTTCAGA-3'

HPV-18 type-specific sequencing primer 5'-GCTTCTACACAGTCTCCTGT-3'

HPV -6 type-specific sequencing primer 5'-GTGCATCCGTAACTACATCTT-3'

HPV -11 type-specific sequencing primer 5'-GTGCATCTGTGTCTAAATCTG-3'

All oligonucleotides used as primers for this study were synthesized and purified with the oligonucleotide purification cartridge (OPC) method by the Pathology Department DNA Synthesis Lab, Yale University (New Haven, CT).

To avoid cross contamination, three separate rooms with no air re-circulation were dedicated to nucleic acid amplification tests. Two of the rooms were each equipped with a 32" PCR workstation (AirClean Systems, Raleigh, NC). All pre-amplification procedures were performed in PCR station I. All post-PCR procedures were carried out in PCR station II, including preparations for the nested PCR and sequencing reaction. Gel electrophoresis and DNA sequencing were performed in the third isolation room. No post-PCR materials or any items contaminated by amplicons, or equipment used in the post-PCR rooms were allowed to enter the pre-PCR working space.

Results

The LoTemp™ HiFi® DNA polymerase was about 10 times more efficient than *Taq* DNA polymerases in amplifying HPV plasmid DNA by MY09/MY11 PCR and about 100 to 1000 times more efficient when the first amplification was followed by a GP5+/GP6+ nested PCR in tandem. The nested PCR technology described in this paper proved to be a sensitive method for the detection of 1–10 copies of purified genomic DNA of HPV types -16, -18 or -6B. But 10^4-10^5 copies of genomic DNA were needed as PCR templates for UV visualization of a positive MY09/MY11 primer amplicon band after electrophoresis (Fig. 1). The specificity of all nested PCR products was validated by HPV genotyping with direct DNA sequencing.

Reproducibility of this nested PCR assay in the detection of HPV DNA in clinical specimens was confirmed by running two parallel sets of PCR with a split single sample digestate as the paired templates, including the β-globin gene, the MY primer and the GP nested primer amplifications on each set for 30 HPV-positive cases. Pairs of identical results on electrophoresis gel were obtained in all three amplifications for the 30 split samples (Fig. 2). The nested PCR products obtained on the duplicate sets were

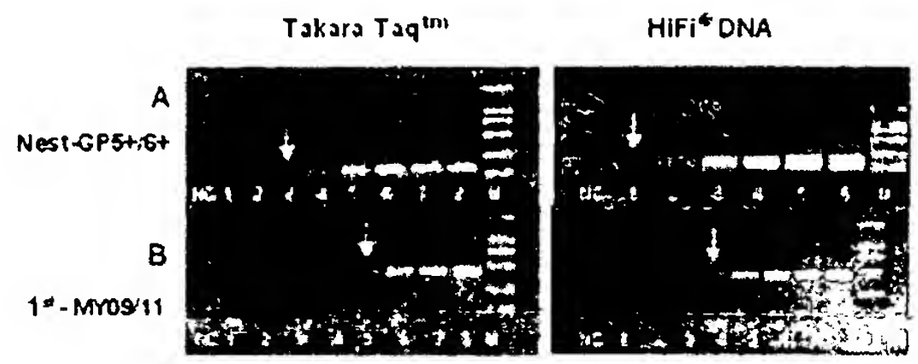


Figure 1
Comparative amplification of HPV-16 DNA with Taq DNA polymerase and with LoTemp™ HiFi®DNA polymerase. Lanes 1–8: HPV-16 plasmid DNA template at 85×10^1, 85×10^2, 85×10^3, 85×10^4, 85×10^5, 85×10^6, 85×10^7 and 85×10^8 copies per milliliter. NC: negative control. M: molecular marker. Arrows indicate the lower limit of detection by nested PCR (A) and by the 1st primary PCR (B) with Takara Taq DNA polymerase and LoTemp™ HiFi® DNA polymerase, respectively.

confirmed by DNA sequencing to be of the same HPV genotype.

For clinical samples, the primary MY09/MY11 PCR generated a distinct 450 bp product band after 30 repeated amplification cycles in only 37 of the 107 HPV-positive cases detected by nested PCR. More than 65% of the clinical HPV-positive amplicons relied on a nested PCR for UV visualization. Without a nested PCR, the amplicon of the MY09/MY11 PCR was often masked due to co-amplification of other DNA molecules in the clinical samples (Fig. 2).

For some isolates, notably those of HPV-39 and HPV-73, the standard GP5+/GP6+ nested PCR failed to amplify the target DNA fragment even when the MY09/MY11 primer PCR amplification was successful. These cases were recognized on the gel electrophoresis, showing a positive 450 bp MY09/MY11 PCR amplicon in the absence of an expected concomitant 150 bp nested PCR product. For these HPV strains, a heminested PCR with MY11/GP6+ primers generated a homogeneous amplicon (Fig. 3) for direct DNA sequencing.

After cycle sequencing, BLAST algorithms by alignment of a 34 bp DNA sequence in the hypervariable region of the L1 gene downstream of the GP5+ binding site against known HPV genotype sequences stored in the GenBank database usually determined the genotype of the HPV isolates detected [32]. A 100% "identities" match between the "query" sequence and the "subject" sequence was reached for each final genotyping (Fig. 4). This sequence

Figure 2
HPV Nested PCR in Clinical Samples and Reproducibility. Agarose gel showing PCR products of targeted DNAs extracted from two clinical samples in duplicate. The targeted β-globin DNA amplicon is 110 bp long, as seen clearly in lanes 25 and 26 (#1210), but is hardly visible in lanes 31 and 32 (#1211). Co-amplification of other human genomic DNA fragments and a positive nested PCR amplicon assure specimen adequacy in both samples. Molecular ruler = 100–1000 bp (far left). Lanes 25/26, 27/28, 29/30 = β-globin gene, MY09/MY11, GP5+/GP6+ PCR, respectively-sample #1210. Lanes 31/32, 33/34, 35/36 = β-globin gene, MY09/MY11, GP5+/GP6+ PCR, respectively-sample #1211

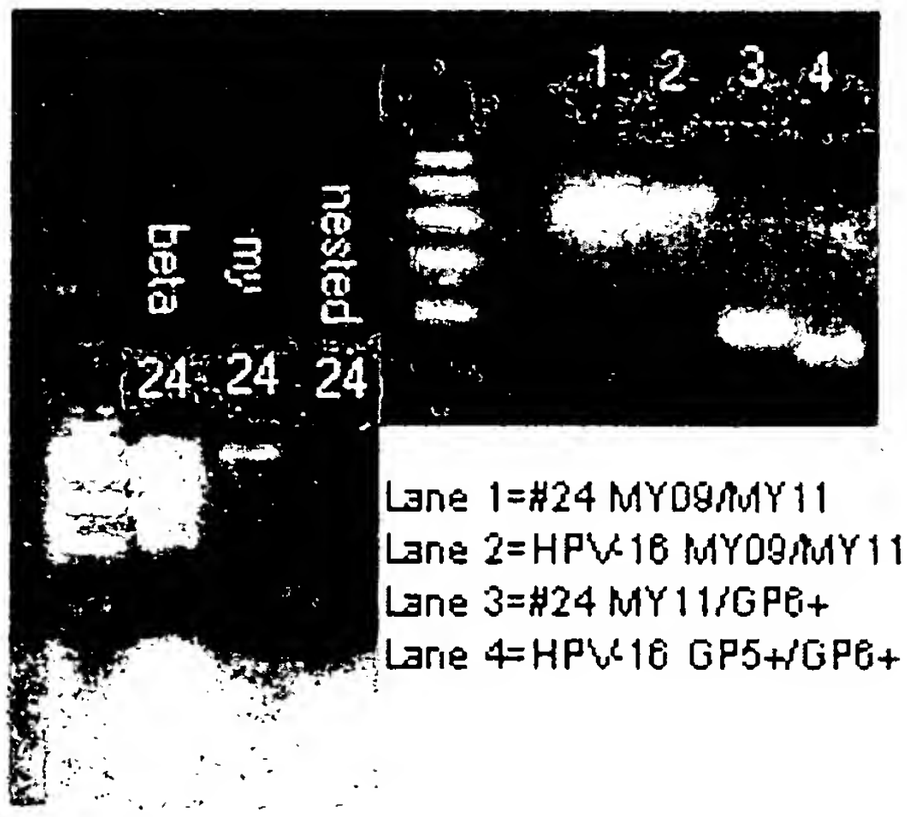
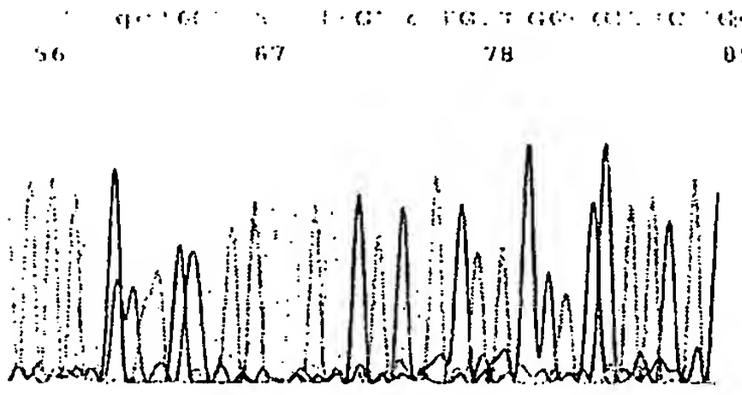


Figure 3
HPV-39 Heminested PCR (clinical specimen #24). Left gel: beta: β-globin gene amplification-specimen #24. my: Positive 450 bp MY09/MY11 PCR product- #24. nested: Negative GP5+/GP6+ nested PCR product- #24. Molecular marker on far left. Right gel: Lane 1: #24 MY09/MY11 PCR product, 450 bp. Lane 2 HPV-16 control Y09/MY11 PCR product, 450 bp. Lane 3: #24 MY11/GP6+ heminested PCR product ~ 195 bp. Lane 4: HPV-16 control GP5+/GP6+ nested PCR ~ 150 bp. Molecular marker on far left



Human papillomavirus type 66 isolate
Length=391

Score = 69.9 bits (35), Expect = 8e-12
Identities = 35/35 (100%), Gaps = 0/35 (0%)
Strand=Plus/Minus

Figure 4
DNA Sequence of Nested PCR Product Downstream of GP5+ for Genotyping. This is a typical DNA sequence excised from the color tracing downstream of the GP5+ binding site of the HPV DNA L1 gene. BLAST alignment analysis of a 34 (up to 50) bp sequence of this hypervariable region provides unequivocal evidence for HPV genotype 66 based on the database stored in the GenBank.

tracing with its on-line BLAST algorithm for genotyping was incorporated in the report for clinical follow-up of persistent infections. However, HPV-16 has numerous sequence variants, some of which share an identical sequence in this region with some strains of HPV-31 and HPV-33, and required BLAST algorithm of a 50 bp sequence for genotyping distinction.

Of the 513 liquid-based cervicovaginal samples, the nested PCR method detected at least one HPV strain in 107, with an overall positive rate of 20.9%, including 74 cases harboring at least one of the 13 types targeted by the Digene HC2 "high-risk" HPV test and 33 cases containing "low-risk" HPV types (Table 1). The most prevalent genotype in the New Haven area was found to be HPV-16, followed by HPV-56. The combined rate of prevalence of HPV-16 and HPV-18 constituted 32.8% of the total isolates.

The HC2 high-risk HPV test identified only 11 (37.9%) of the 29 HPV-16 cases as positive. However, it successfully identified all the samples containing HPV-56 and HPV-18 as positive for high-risk HPV. The sensitivity of the HC2 test in detecting the predetermined "high-risk" HPV genotypes was summarized as follows:

HPV-18 100%

HPV-33 100%

HPV-39 100%

HPV-56 100%

HPV-59 100%

HPV-68 100%

HPV-58 83.3%

HPV-31 71.4%

HPV-45 66.7%

HPV-35 50%

HPV-52 50%

HPV-16 37.9%

Although HPV-54, -66, -83, -53 and -62 were not included in the hybridization cocktail probe, these genotypes were often reported to be positive for high-risk HPV by the HC2 test (Table 1).

Among the 107 nested PCR-positive samples, DNA sequencing with the GP6+ consensus general primer yielded multiple overlapping unreadable sequences in 5 cases. Using the individual type-specific primer sequencing for HPV-6,-11,-16 and -18 proved that one of them contained HPV-16, but not the other three genotypes, and that one contained a mixture of HPV-16 and HPV-18, but not the other two genotypes. For the remaining 3 mixed infection samples, repeated individual DNA sequencing failed to produce a readable primer extension/termination reaction with any of the four type-specific primers. Therefore, these latter 3 cases were considered to be multiple infections caused by HPVs other than the four vaccine-relevant types and grouped under the "low-risk" category.

Of the 513 cases studied, the Digene HC2 test classified 403 samples as negative and 75 as positive for "high-risk" HPV, and 35 as unsatisfactory for evaluation. These results were compared with those obtained by the nested PCR assay (Table 2). Since the Digene test only covered the "high-risk" HPV genotypes, specimens infected by HPV other than the 13 types targeted by the HC2 "high-risk" HPV cocktail probe would be classified as negative or unsatisfactory for evaluation (unsat.). In summary, HC2 test identified 50 of the 74 "high-risk" HPV-positive sam-

Table 1: HPV genotyping by DNA Sequencing v. Digene HC2 Test

PCR/DNA Sequencing			*Test Results by Digene HC2*	
Type Positive Cases		Prevalence (%)	High-risk+	Negative
16	29	(27.2)	11	18
56	9	(8.5)	9	0
31	7	(6.5)	5	2
6	6	(5.6)	2	4
18	6	(5.6)	6	0
54	6	(5.6)	3	3
58	6	(5.6)	5	1
66	6	(5.6)	6	0
59	4	(3.7)	4	0
45	3	(2.8)	2	1
83	3	(2.8)	2	1
32	2	(1.9)	0	2
35	2	(1.9)	1	1
39	2	(1.9)	2	0
40	2	(1.9)	0	2
52	2	(1.9)	1	1
33	1	(0.9)	1	0
53	1	(0.9)	1	0
62	1	(0.9)	1	0
68	1	(0.9)	1	0
70	1	(0.9)	1	0
72	1	(0.9)	0	1
73	1	(0.9)	0	1
M16	1	(0.9)	1	0
M 16, 18	1	(0.9)	1	0
M others	3	(2.9)	1	2
Total	107	(100)	67	40
			HC2 HPV detection rate = 67/107 = 62.6%	
Samples 513				
%HPV+ 20.9				

M16 = mixed infection with HPV 16 identified by type-specific sequencing primer.
M 16, 18 = mixed infection with HPV 16 and HPV 18 identified by type-specific sequencing primers.
M others = mixed infections by HPV types which cannot be sequenced with type-specific primers for HPV 6, 11, 16 or 18.
Digene High-risk + includes those cases reported as positive for both High-risk and Low-risk HPV types.
The underlined HPV genotypes are included in the "High-risk" Digene HC2 cocktail probe.

ples detected by nested PCR. The analytical sensitivity of the Digene HC2 test was calculated to be 50/74= 67.6% against the nested PCR assay. Since HC2 reported a total of 75 cases to be positive for "high-risk" HPV, its analytical specificity was calculated to be 50/75 = 66.7%. Two cases found to be unsatisfactory for PCR evaluation and negative by HC2 test were excluded from the total of 513 cases entered for analysis.

Discussion

The moderately thermostable LoTemp™ HiFi® DNA polymerases are genetically engineered derivatives of a *Bacillus stearothermophilus (Bst)* DNA polymerase which was first introduced to resolve the hairpin structure in classic Sanger DNA sequencing by Ye and Hong [33]. *Bst* DNA polymerase with an optimum primer extension temperature at 65°C is extremely stable, capable of retaining its sequencing quality in working solution at room temperature for several weeks under conditions of robotic automation [34]. Modification of the amino acid sequence of a natural *Bst* DNA polymerase by site-directed genetic mutations increases the heat tolerance of the enzyme [35], which has paved the way to development of new thermostable DNA polymerases for repeated primer extension reactions under 85°C. The LoTemp™ HiFi® DNA polymerase PCR protocol exploits the proof-reading and high-processivity properties of the modified *Bst* DNA polymerases at reduced cyclic temperatures.

Using LoTemp™ HiFi® DNA polymerase for HPV DNA amplification eliminates all template purification steps which are usually required before PCR or sequencing reaction [21,22,32]. Since the ready-to-use polymerase mixture contains all the required ingredients for PCR, the need for in-house pipetting is minimal. Since the DNA polymerase and other reagent components are stabilized for storage at room temperature, there is no need to keep ice-cold blocks while setting up the PCR. Since this system uses chemical melting agents for dsDNA denaturing and a high-processivity DNA polymerase for nucleotide primer extension under partial isostabilization, it allows thermocycling at 85°C for denaturing, 40°C for annealing and 65°C for primer extension, respectively. At lowered cycling temperatures, the rate of heat-induced mutations [36], namely depurination [37] and deamination [38] of the nitrogenous bases, in the DNA molecules during PCR amplification is reduced. As a result, the PCR products are more homogeneous.

In this report, we have demonstrated that the LoTemp™ PCR system can amplify 1–10 copies of purified HPV-16, -18 or -6B to generate a corresponding type-specific 150 bp nested PCR product. The sensitivity of LoTemp™ PCR in the detection of plasmid HPV DNA is about 10× greater than that obtained by the traditional heat-resistant *Taq* DNA polymerases without nested amplification (Fig. 1). Nested PCR has increased the analytical sensitivity of HPV DNA detection on cervicovaginal cell suspensions [21,22,32], which is also our experience (Fig. 2). Nested PCR also serves to eliminate most of the interfering substances which otherwise may have to be removed by column purification in the procedure.

Although the MY09/MY11 and GP5+/GP6+ consensus general primer pairs have been used to amplify the highly conserved L1 gene region of all HPV genotypes, their efficiency in target DNA amplification varies from one HPV

Table 2: Comparison of HPV nested PCR/sequencing and HC2 test results

		PCR Results				
		Negative	**High Risk**	**Low Risk**	**Unsat.**	**Total**
HC2 Results	**Negative**	364	23	16	0	403
	High Risk	8	50	17	0	75
	Low Risk	NP	NP	NP		
	Unsat.	34	1		0	35
	Total	406	74	33	0	513

HC2, Hybrid Capture 2; **HPV**, human papillomavirus; **PCR**, polymerase chain reaction.
Unsat. Test results or specimens considered inadequate for evaluation.
High Risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68.
Low Risk HPV types other than those listed as High Risk.
NP = not performed.
Data are given as number of specimens

genotype to another with discordant patterns [22]. We have found that some isolates of HPV-39 and HPV-73 are not amplifiable by the GP5+/GP6+ primer pair either in primary PCR or in nested PCR. A second amplification with a GP5+/MY09 and a GP6+/MY11 primer pair generates a longer and shorter PCR product, respectively. The GP6+/MY11 heminested PCR product (Fig. 3) is suitable for direct DNA sequencing. GP5+/GP6+ failure in amplification of HPV-39 [39] and other HPV types [22,32] in clinical specimens is a well-known technical problem if this single pair of primers is relied upon for HPV detection. However, GP5+ and GP6+ are excellent nested PCR primers for preparing templates for HPV genotyping with direct DNA sequencing.

Optimization of the PCR protocol is essential for detection of HPV. Without optimization, a PCR method using one set of consensus primers for amplification may have a lower sensitivity than the Digene HC2 test [40]. Using an initial 40-cycle amplification on clinical specimens, Johnson *et al.* [21] reported that nested PCR generally increases the sensitivity of an HPV PCR method by about 60%. With the PCR protocol presented in this paper, this difference is augmented due to adoption of a 30-cycle amplification. An advantage of reducing the number of amplification cycles is that co-amplification of non-specific interfering DNA molecules is reduced in the primary PCR in favor of generating specific nested PCR products for direct DNA sequencing.

Using our optimized protocol, nested PCR detected 107 HPV-positive cases among 513 clinical samples (Table 1). Twenty-three (23) HPV genotypes have been identified, including 12 of the 13 "high-risk" genotypes, i.e. HPV-16, -18, -31, -33, -35, -39, -45, -52, -56, -58, -59, and -68, which are targeted by the Digene HC2 test. The lack of representation of HPV-51 which is also targeted by the HC 2 test probably reflects a low regional prevalence of this genotype. Using the same primers for nested PCR followed by genotyping with DNA sequencing, HPV-51 was found to be a relatively common genotype in Germany, constituting about 5% of the total HPV isolates detected [22]. However, it was not recorded even once among 894 HPV isolates in Denmark [21].

The hypervariable region of the DNA sequence downstream of the GP5+ binding site is critical in L1 genotyping of HPV. While most HPV genotypes can be determined through alignment of a 34-bp sequence in this region with the GenBank database [32], accurate genotyping may require alignment of a longer sequence, for example 50 bp long, when the result of BLAST algorithm is ambiguous. If a 34-bp sequence is relied upon for genotyping, the number of HPV-16 infections may be underestimated.

For the development of this protocol, we found that using 1 μL of 5 μmolar OPC-purified GP6+ oligonucleotide instead of a manufacturer-recommended 3.2 μmolar solution as the sequencing primer seems to generate more consistent sequencing results for typing various HPV isolates. The concentration of primer may need to be adjusted if a higher grade of GP6+ is used as the DNA sequencing primer.

Of the 107 PCR-positive samples, the Digene HC2 test has classified 67 as HPV-positive, a detection rate of 62.6% (Table 1). When only the HC2-targeted "high-risk" HPV genotypes are used for comparison, nested PCR detects 74 HPV-positive cases while HC2 test identifies 50 in this group with an analytical sensitivity of 67.6% (50/74).

The most prevalent is HPV-16, constituting 27.2% of the total isolates (Table 1). This percentage of HPV-16 prevalence is almost identical to those reported by others using MY/GP nested PCR/DNA sequencing genotyping on cervicovaginal cell suspensions, e.g. 26% in Denmark [21] and 26.2% in Germany [22]. Digene HC2 test identified 11 of

the 29 HPV-16 PCR-positive cases with a detection rate of 37.9% which is surprisingly low. Since all the HPV-16 PCR-positive results have been validated by DNA sequencing for genotyping with a prevalence rate similar to those commonly found in the Western world based on the same methodology [21,22] and since the Digene HC2 tests were performed by two independent clinical laboratories properly certified by the health authorities, the validity of the individual test results seems not to be in question. The discrepancy is probably due to the fact that there are numerous HPV-16 sequence variants in a given patient population [41], which may not be all targeted by the HC2 RNA cocktail probe, but share a highly conserved region of the L1 gene that the MY09/MY11 primers amplify effectively. In support of this interpretation is the fact that the HC2 test has correctly identified all cases of HPV-18, HPV-39, HPV-56 and HPV-59 as "high-risk" in the same data.

HPV-56 is the second most prevalent high-risk genotype detected (8.5%), followed by HPV-31, -18, -54, -58 and -66, sharing about the same rate of prevalence (5.6–6.5%). The combined number of HPV-16 and -18 cases constitutes only 32.8% of the single HPV isolates in our series. HPV-18 also seems to play a relatively minor role in causing cervical pathology in Canada [42].

It has been reported by others [43] that the Digene HC2 high-risk test may be able to detect HPV types -53, -54, -62, -66 and -83 and label them as high-risk HPVs although these genotypes are not intentionally targeted in its high-risk cocktail probe. Our findings confirm these cross-reactions (Table 1). Sequence variation within the probe binding sites [44] and non-specific binding between the probe and non-targeted mismatched DNA [45-48] are well recognized sources of error if nucleic acid hybridization is relied upon for microbial and viral genotyping. When the GP5+/GP6+ PCR products with a hypervariable DNA sequence are targeted for developing a multiplex genotyping method [49], the DNA probe designed for HPV-66, a recently recognized high-risk type [50-52], is found to react with HPV-52 and the probe for HPV-82 with HPV-51 due to cross-hybridization despite the presence of four base mismatches in each pair. Some experts [40] consider the unintended cross-reactions of the Digene HC2 test with non-targeted HPV types, such as HPV-66, which occasionally may cause cancer, to be "fortunate". However, the benefit to the patients of these cross-reactions needs additional confirmation.

Fifty percent (50%) of the HPV-54 isolates are returned by HC2 test as high-risk HPV. HPV-54 has been classified as a low-risk virus [51,53], but is found to be associated with a 40-fold increase in risk among American Indian women with CIN 2/3. Only HPV-16 has shown a higher risk than HPV-54 among this subpopulation [54]. Genetic make-up of a patient may have to be considered in using HPV genotyping information for the follow-up of persistent infections [55].

For the mixed infection cases, we choose single primers specific for HPV-6, -11, -16 and -18 [31] to perform individual specific primer DNA sequencing in order to determine if the mixed infection includes any of these vaccine-relevant HPV types. The rationale for this choice is that the majority of multiple HPV infections are transient [6-15]. The immediate concern to the patient and her health care provider is whether the mixed infection is caused by any of the vaccine-relevant HPV types if the patient is considering vaccine immunization.

The low percentage (< 5%) of multiple HPV infections observed in our series might have been biased because a large proportion of the specimens for this study was collected from a solo private practitioner's office and this group of specimens had an exceptionally low positive rate and no multiple HPV infections at all. The rate of multiple HPV infections is known to vary among patient populations and is also influenced by the stage of carcinogenesis. Multiple HPV infections were found in less than 5% of the HPV-positive samples from patients with invasive cancer lesions, but over 15% of the positive samples in the control group [27]. Multiple HPV infections tend to evolve into single HPV infections as the infection becomes chronic and persistent while the cervical cytopathology progresses from metaplasia, LSIL, HSIL, carcinoma-in-situ to invasive cancer [56].

In summary, we have reported our experience in adapting the well characterized PCR/DNA sequencing protocol for routine HPV genotyping. We believe that a sensitive and specific HPV assay followed by genotyping with direct DNA sequencing will generate useful information for following persistent infections while referring the patients at "high-risk" of developing HSIL, not the patients with a "high-risk HPV", to colposcopic evaluation.

Conclusion

The nested PCR technology using MY09/MY11 and GP5+/GP6+ consensus primers for target HPV DNA amplification can be used in diagnostic laboratories for routine HPV detection and to prepare clinical materials for genotyping by direct DNA sequencing. We have adapted a newly introduced low-temperature PCR system and optimized the protocol to facilitate the transfer of this molecular technology into clinical laboratory practice for accurate HPV genotyping which is a valuable tool for follow-up of patients with persistent HPV infection, a recognized tumor promoter in cancer induction.

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